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University of Minnesota	May 28, 1947
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Louisiana State University	May 9, 1947
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15892

Apparent Serological Variation Within a Strain of Influenza Virus.*

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During the period of testing preparations of influenza virus for comparative immunizing effect in mice, certain results were encountered suggesting that the PR8 strain of Type A influenza virus maintained in eggs had lost antigenic potency when measured by its capacity to protect mice against the mouse passage line of the same strain. The egg line had been transferred to tissue culture in 1935 from the 70th mouse passage and had been continued in culture for 717 transfers until January, 1942, when it was introduced into fertile hens' eggs. From that time until March, 1944, when the present observations were begun, it had received 53 transfers in eggs. Because of this effect a new line of the PR8 strain was instituted in eggs from the 593rd unbroken mouse passage line. A series of studies was then undertaken to compare the immunological behavior of the 2 lines.

Materials and Methods. Virus. The virus is the PR8 strain of influenza virus, Type A, isolated in 1934 by the inoculation of ferrets. After 7 ferret passages it was transferred to mice. The mouse line was thereafter constantly maintained in mouse passage only. The tissue culture line was established from the 70th mouse passage in 1935. It was then maintained by transfers done twice weekly on the average for 717 generations in culture, until it was transferred to eggs in 1941.

Serological Tests. (a) Hemagglutination and inhibition: This procedure was carried out with a pattern test[1] in which one unit of agglutinin was employed for the titration of serum. Results are given as final dilutions of serum.

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* This investigation was conducted under the auspices of the Commission on Influenza, Army Epidemiological Board, Office of the Surgeon General, U. S. Army, Washington, D.C.

[1] Salk, J. E., *J. Immunol.*, 1944, **49**, 87.

TABLE I.
Cross-Immunity Tests in Mice.

	3/28/44 Infection with mouse passage						
Immunized with	10^{-1}	10^{-2}	10^{-3}	10^{-4}	Protection LD_{50}	Hemagglutinating titer	Mouse infection titer
TC-Egg							
10^{-0}	5/7	6/6	6/6	6/6	>100,000	2560	$10^{-5.15}$
10^{-1}	2/7	5/6	6/6	6/6	56,230		
10^{-2}	1/6	3/7	5/6	6/6	10,890		
10^{-3}	1/6	0/6	3/7	3/6	560		
10^{-4}	0/6	0/6	0/7	4/6	250		
Mouse-Egg							
10^{-0}	3/3	—	—	—	>100,000	1280	10^{-6}
10^{-1}	7/7	6/6	6/6	6/6	>100,000		
10^{-2}	5/6	6/6	6/6	6/6	>100,000		
10^{-3}	1/6	7/7	6/6	6/6	56,230		
10^{-4}	0/6	6/6	6/6	6/6	45,390		
Control	10^{-5}	10^{-6}	10^{-7}		LD_{50}		
Titration	0/5	3/5	1/5		1,197,000		

Numerator = Survivors. Denominator = Number inoculated.

(b) Neutralization in mice: For this test the virus suspension and serum dilution were mixed, allowed to stand at room temperature for 30 minutes and each of a group of 3 to 5 mice was given 0.03 cc of a mixture intranasally. The mice were observed for 10 days and the endpoint was recorded as the estimated point of 50% lethality.[2] The dilutions of serum employed varied according to the range of neutralizing titer anticipated in the system. Titers are recorded as final dilutions of serum.

(c) Titrations of virus potency in mice were ordinarily done with serial 10-fold dilutions.

(d) Rabbit sera were generally obtained 10 days after one intraperitoneal inoculation of active virus preparation. In some instances the use of hyperimmune rabbit serum is indicated; animals received multiple inoculations of virus.

Egg Passage. For passage of virus in eggs, inoculations of 0.1 cc volume were made directly into the allantoic sac of 10-day embryos. After 48 hours' incubation at 37°C, the fluid was harvested with needle and syringe in the absence of hemorrhage. Material was titrated for hemagglutinins and, in stoppered containers, was stored in the refrigerator at 4°C until used.

Cross Immunity in Mice. The 2 lines, PR8, 198-M70, TC717-E55, and PR8, 198-M593-E4, were used. Allantoic fluid was harvested from chick embryos inoculated 48 hours earlier. Titers of hemagglutinin were determined with each preparation at the time of harvesting. The fluid was kept at 4°C in the intervals between use. Each of a group of 30 to 35 mice was given 2 intraperitoneal injections of 0.5 cc amounts of undiluted or 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} dilutions of one of the 2 preparations at weekly intervals. Titrations of intranasal infectivity for mice were made at the time of each immunizing inoculation.

One week after the second inoculation each group of mice was subdivided into 5 subgroups and these were inoculated intranasally with 10^{-1}, 10^{-2}, 10^{-3} or 10^{-4} dilutions, respectively, of suspension of lungs of mice of the 597th regular passage of PR8 strain. Inoculated control mice of the same lot were infected at the same time.

The mouse-egg line was approximately 10 times more effective in its lethal titer for mice, but the tissue culture-egg line had a somewhat higher titer of hemagglutinins. As measured by immunizing effect against regular mouse passage virus, the mouse-egg line was at least 100 times as effective as the tissue culture-egg line. The effect was emphasized by the fact that in the same test 2 lines of the Weiss strain were also studied, one transferred to eggs directly from ferret passage, the other after 32 mouse passages. The former ordinarily produced fatal infection in mice only with undiluted fluid while the latter had a lethal titer of 10^{-4} approximately. Both lines induced essentially the same degree of active immunity against the PR8 mouse passage virus, somewhat more effectively than the PR8 tissue culture-egg line.

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[2] Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

The results were confirmed by a later limited experiment with the 77th egg passage of the tissue culture-egg line and the 11th egg passage of the mouse-egg line. The titers of the 2 preparations were, respectively, by hemagglutination 2560 and 1280; by intranasal infection of mice, $10^{-5.3}$ and $10^{-5.5}$; by infection of fertile eggs allantoically, $10^{-8.48}$ and $10^{-8.41}$. The undiluted tissue culture-egg material protected against 100,000 LD_{50}, the 10^{-1} dilution against 21,000 and the 10^{-3} dilution against less than 1400. The 10^{-1} dilution of the mouse-egg material induced immunity against more than 150,000 and the 10^{-3} dilution against 100,000 LD_{50} of the regular mouse passage virus. Hence, while the virus content of the 2 preparations was closely similar, the immunizing disparity persisted.

The data, in review, suggested that antigenic differences existed between the 2 lines of the PR8 strain maintained under different conditions for several years or that cultivation in eggs had an effect upon virus behavior.

Serological Studies. Experiment I. Rabbit RM 3 received 5.0 cc intraperitoneally of the allantoic fluid containing the tissue culture-egg line in its 54th passage. Rabbit RM 4 received 5.0 cc intraperitoneally of the mouse-egg line in its second egg passage. Serum was obtained from both animals before inoculation and 10 days afterwards. The sera were then used in agglutination-inhibition tests against eluates of the 2 lines and in neutralization tests in mice with approximately 100 LD_{50} of the tissue culture-egg

TABLE II. Serological Tests with Different Lines of Virus.

	Rabbit No.	Date of serum	Stage	Immunized with strain	Passage	Agglutination-inhibition test		Neutralization in mice	
						TC-Egg 43 eluate	Mouse-Egg 3 eluate	TC-Egg 57 10^{-2}	Mouse 597 10^{-3}
Exp. I	RM 3	3/22/44	Norm.			128	64	0	0
		4/ 1	Imm.	TC-Egg	54	512	512	100	8
	,, 4	3/22	Norm.			128	32	0	0
		4/ 1	Imm.	Mouse-Egg	2	2048	4800	280	480
		8/17	Imm.			1024	2048		
						TC-Egg 77 eluate	Mouse-Egg 11 eluate	TC-Egg 77 10^{-1}	Mouse-Egg 11 10^{-2}
Exp. II	RM 12	10/ 9	Norm.			64	32	10	10
		10/27	Imm.	Mouse-Egg	20	1024	2048	140	960
								640	1600
	,, 16	11/22	Norm.			32	32	0	0
		12/ 2	Imm.	Mouse	634	1024	1024	40	480
	,, 21	11/22	Norm.			32	32	10	10
		10/27	Imm.	TC-Egg	75	4096	512	70	35
								280	30

Exp. I: Mouse titer of TC-Egg strain $10^{-4.25}$, 10^{-2} used. Mouse titer of Mouse strain $10^{-6.25}$, 10^{-3} used. Exp. II: Mouse titer of TC-Egg strain $10^{-5.33}$, 10^{-1} used. Mouse titer of Mouse-Egg strain $10^{-5.56}$, 10^{-2} used. Egg titer of TC-Egg strain $10^{-8.5}$; of Mouse-Egg strain $10^{-8.4}$.

and approximately 1000 LD_{50} of the regular mouse passage lines. Equal volumes of 2-fold dilutions of serum and the virus preparation were mixed, incubated for 30 minutes and each mixture was then given intranasally in 0.05 cc amounts to 3 mice.

The results of the agglutination-inhibition test, presented in Table II, gave only an indication that the serum of the rabbit injected with mouse-egg line had a higher titer against the homologous than against the heterologous line. On the other hand, in the neutralization test in mice, each serum gave higher protection against its homologous line although the lethal dosage of mouse-lung virus employed was greater than that of the tissue culture-egg line.

Experiment II. Rabbit RM 12 received intraperitoneally 5.0 cc of allantoic fluid from the 20th egg passage of mouse-egg line. Rabbit RM 16 received intraperitoneally 5.0 cc of suspension of lungs of mice in the 634th passage. Rabbit RM 21 received intraperitoneally 5.0 cc of allantoic fluid from the 75th egg passage of the tissue culture-egg line. They were tested by agglutination-inhibition and neutralization in mice against a 10^{-1} dilution of virus in allantoic fluid from the 77th egg passage of the tissue culture-egg line and against a 10^{-2} dilution of fluid from the 11th egg passage of the mouse-egg line. These materials described in the second immunization test in mice had closely similar infectious titers in eggs and mice.

In this instance the agglutination-inhibiting test was again inconstant in pointing out differences between the lines. The sera of rabbits injected with the mouse-egg and the mouse materials gave much higher neutralization of the homologous mouse-egg line than of the tissue culture-egg line. The latter was neutralized to a higher titer by the homologous serum.

Time of Occurrence of Deviation. Since an obvious explanation of the observed differences is that a contamination of the PR8 strain or a substitution had occurred, tests were made with sera against strains which had been employed in this laboratory in recent years. No sharp differences in neu-

TABLE III.

Serological Tests with Sera Against Different Strains of Virus.

Date of Test 1/9/45.

Rabbit No.	Date of serum	Stage	Immunized with strain	Passage	Agglutination-inhibition test		Neutralization-in-mice	
					TC-Egg	Mouse-Egg	TC-Egg-Mouse	Mouse or Mouse-Egg
R 204	5/21/37	Imm.	Melbourne	217-7			<10	<10
RN 47	10/ 2/39	”	W.S.				<20	<20
” 64	4/ 5/41	”	Baum	M-19			<20	<20
RM 5	3/22/44	Norm.			128	64	0	0
	4/ 1	Imm.	Weiss	M-32, E-11	128	512	15	5
” 6	3/22	Norm.			128	128	0	0
	4/ 1	Imm.	Weiss	E-17	512	1024	12	5
R 215	5/ 3/37	Imm.	PR8	M-193			18	50
RN 5	1/18/39	Hyper-Imm.	PR8	M-356-360			240	>320
” 56	10/14/40	Imm.	PR8	M-432			<20	60
RM 16	12/ 4/44	”	PR8	M-634			35	280
12	10/27	”	PR8	M-E-20			640	1600
21	12/ 4	”	PR8	TC-E-75			280	30
RN 3	2/ 6/39	Hyper-Imm.	PR8	TC			80	30
” 60	2/ 1/41	Hyper-Imm.	PR8	TC			120	50

Mouse titer of Mouse passage virus $10^{-6.5}$, 10^{-3} used. Mouse titer of Tissue Culture-Mouse virus $10^{-5.5}$, 10^{-3} used.

tralization tests with the 2 lines were observed with sera against Melbourne, W. S., Baum or Weiss strains. But when tests were made with sera prepared against PR8 mouse lungs as far back as 1937 or sera available against the tissue culture strain since 1939, it was observed that sera against the mouse line gave better neutralization against the homologous line even though comparatively more mouse virus than tissue culture-egg virus was used in the tests. Sera against the tissue culture line also showed higher titers with the homologous line. It was evident that the difference between the lines had been present for some time.

Tests with Human Sera. Both hemagglutination-inhibiting and mouse neutralization tests were done with sera of human individuals who underwent infection with influenza A in 1943 and of others who received influenza virus vaccine, which probably contained the tissue culture-egg line. The results demonstrated sharply that much higher titers were obtained against mouse line than against tissue culture-egg line with postvaccinal or convalescent sera in both tests while differences in the low titers of acute phase or prevaccinal sera were insignificant. The data clearly indicate that the mouse line was much more readily neutralized by immune sera of human individuals than was the tissue culture-egg line. Differences of 4- to 8-fold were seen in the inhibition tests and from 4- to 10-fold in the neutralization test. It is obvious that differences in serological behavior of this extent could seriously influence impressions gained as to the nature of an epidemic strain.

Discussion. The reported observations clearly indicate that variation in antigenic behavior took place in the line of the PR8 strain of influenza virus, Type A, maintained continuously for a period of years in tissue culture and eggs. It appears that the change from the mouse passage line occurred early in its history outside the mouse. It is interesting in this respect that from the earliest studies with the virus in tissue culture, indications were presented[3,4] that some difference between the virus so maintained and the parent strain existed; and it is likely that lack of refinement in procedures may have prevented an earlier clear-cut demonstration. The evidence supports the conclusion that the difference is not due to a replacement of one strain by another.

Burnet and Bull[5] have reported observations suggesting antigenic variation in the egg passage line of the Melbourne strain, and Stuart-Harris[6] has noted changes in titer obtained with sera tested against the PR8 strain after its adaptation to eggs.

The differences described in the present paper are sufficient to constitute an obstacle to proper evaluation of immunity induced by one line and tested with the other.

Summary. Evidence is presented that antigenic variation took place in a line of the PR8 strain of influenza virus propagated through continuous passage in tissue culture and eggs as compared with a line of the same strain maintained in mice.

The author acknowledges with pleasure the assistance of Miss Elva Minuse in the technical procedures.

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[3] Francis, T., Jr., and Magill, T. P., *Science*, 1935, **82**, 353.

[4] Magill, T. P., and Francis, T., Jr., *J. Exp. Med.*, 1936, **63**, 803.

[5] Burnet, F. M., and Bull, D. R., *Australian J. Exp. Biol. and Med. Sci.*, 1944, **22**, 173.

[6] Stuart-Harris, C. H., *Brit. J. Exp. Path.*, 1943, **24**, 33.

15893

Regulation of Pituitary Gonadotrophic Secretion:* Inhibition by Estrogen or Inactivation by the Ovaries?

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The concept that the normal level of circulating estrogen inhibits pituitary gonadotrophic secretion has remained unchallenged long enough to have become the traditionally accepted explanation for control of the ovarian-pituitary axis. It is invoked to explain the rise of gonadotrophins following ovarian extirpation. It is also invoked to explain that the effect of administering estrogens is to suppress pituitary gonadotrophic secretion.

This view was questioned by Lauson, Heller and Sevringhaus[1] upon finding that α-estradiol administered in moderate doses did not prevent the postcastration rise of gonadotrophins in rats. Soon after it was found that doses of estrogen sufficient to alleviate menopausal symptoms and to restore the vaginal and endometrial histological appearance to normal did not lower the postmenopausal rise of urinary gonadotrophins in women.[2] The failure of physiological amounts of estrogen to suppress gonadotrophins was confirmed in castrated women.[3] Not only does substitution of female sex hormones in the female fail to suppress gonadotrophins but also substitution of physiological amounts of androgens in the male fails to suppress gonadotrophins. This has been noted in castrated male rats,[4] in the male climacteric,[5] and in eunuchoids.[6] These data do not substantiate the hypothesis that the normal level of circulating estrogen inhibits pituitary gonadotrophic secretion. (We recognize and have confirmed the fact that unphysiologically large doses of estrogens and androgens have a markedly inhibiting effect upon pituitary gonadotrophic potency).

Since the hypothesis of inhibition by estrogen proves to be inadequate in several situations, an alternative explanation is required. A tentatively satisfactory hypothesis is that in stimulating gonadal growth and secretion, gonadotrophins are altered by the ovary to such a degree that they become inactive. Seidlin's observations[7] and those of Heller, Heller and Sevringhaus[2] strongly suggest this possibility.

Data are presented in this communication indicating that the rise in gonadotrophins following castration is principally due to lack of gonadotrophin inactivation, and only in part due to lack of inhibition by estrogen.

The concept that a target-organ inactivates the specific hormone stimulating it is not entirely without precedent. An analogous situation was postulated for the thyroid-pituitary axis by Loesser[8] and substantiated by Seidlin.[7] It remained for the classical work of Rawson, Sterne and Aub[9] to con-

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We wish to thank Albert Segaloff for his technical assistance in the early stages of the investigation.

[1] Lauson, H., Heller, C. G., and Sevringhaus, E. L., *Endocrin.*, 1938, **23**, 479.

[2] Heller, C. G., Heller, E. J., and Sevringhaus, E. L., *Endocrin.*, 1942, **30**, 309.

[3] Heller, C. G., Chandler, R. E., and Myers, G. B., *J. Clin. Endocrin.*, 1944, **4**, 109.

[4] Heller, C. G., Segaloff, A., and Nelson, W. O., *Endocrin.*, 1943, **33**, 186.

[5] Unpublished data.

[6] Heller, C. G., Nelson, W. O., and Roth, A. A., *J. Clin. Endocrin.*, 1943, **3**, 573.

[7] Seidlin, S. M., *Endocrin.*, 1940, **26**, 696.

[8] Loeser, A., *Arch. exp. Path. u. Pharmakol.*, 1933, **173**, 62.

[9] Rawson, R. W., Sterne, G. D., and Aub, J. C., *Endocrin.*, 1942, **30**, 240.

TABLE I.

		Donor rats							Recipient rats*		
		Before autopsy			At autopsy				Uterine wt		
		Vaginal smears		Ovarian wt							
	No. of rats	Before operation	After operation	At operation mg	At autopsy mg	Ovarian appearance	Uterine appearance	Thymic wt, mg	With fluid, mg	Without fluid, mg	Ovarian wt, mg
Intact controls	22	cycling	cycling	—	49.9	normal	normal	154	162	89	10.3
Castrate ,,	29	,,	atrophic	normal	—	—	all atrophic	290	92	92	92.4
Ovary-spleen† non-adhesion	25	,,	,,	49.0	149.5	homogenous corpora lutea	all atrophic	235	123	94	20.4
Ovary-spleen adhesion	24	,,	cycling	50.5	32.9	normal	normal§	156	163	97	28.8
Ovary-spleen adhesion and non-adhesion 0.5-5.0 μg/day estradiol benzoate	26	,,	estrus	62.3	14.9	atrophic	all estrus	132	53	45	11.2
Ovary-spleen adhesion and non-ahdesion 0.25-0.05 μg/day estradiol benzoate	13	,,	cycling‡	48.0	21.5	normal	normal§	168	168	92	22.3
Control wts of assay animals									30	30	10.0

* Each recipient rat was injected with a suspension made from a single donor rat pituitary.

† Both ovaries were removed and autotransplanted to the spleen.

‡ Cycles were irregular.

§ Normal signifies that various stages of stimulation were encountered (from diestrus to estrus).

clusively demonstrate that thyroid and lymphoid tissues and not other tissues growing *in vitro,* were capable of inactivating thyrotrophic hormones.

Materials and Methods. Adult virgin female rats of the Sprague-Dawley strain, weighing 200-250 g, were divided into 6 donor groups as listed in Table I. Autotransplants were made by placing both ovaries in the spleen during a single operative procedure in the experimental groups.

Donor pituitaries were macerated, suspended in saline and injected twice daily subcutaneously into 22-24-day-old immature female rats of the same strain for 3 days. Autopsies of recipients were made 24 hours later. One donor gland was injected into one recipient rat.

Results are listed in Table I.

Discussion. Regulation of pituitary gonadotrophin secretion in *normal intact animals* (Fig. 1) could be accomplished by either (or both) of 2 mechanisms: (a) Inactivation of gonadotrophins incident to their usage by the ovary could lead to lowering of circulating gonadotrophins (at position 1 in the figures). (b) The level of circulating estrogen could determine the amount of pituitary gonadotrophic secretion; *i.e.,* as the estrogen level increases gonadotrophin secretion decreases and vice versa (at position 4 in the figures).

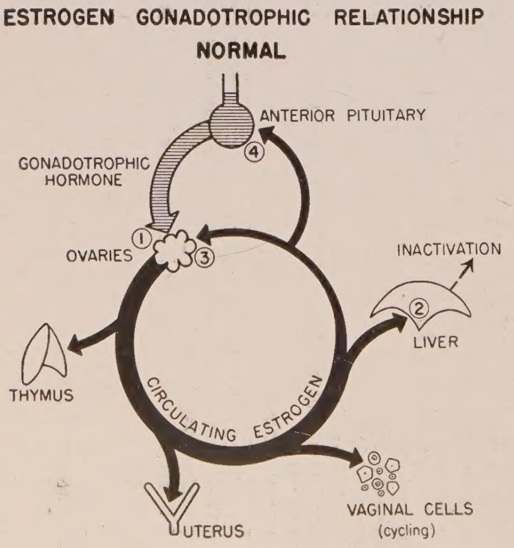

Fig. 1.

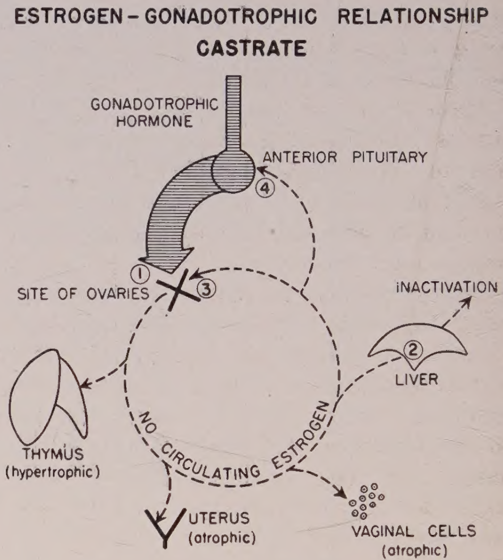

Fig. 2.

The rise in pituitary gonadotrophic potency from 10.3 mg (weight of ovaries of recipient assay animals) for the intact controls to 93.4 mg for the *castrate controls* (Fig. 2) can be explained by either or both of the 2 hypotheses: (a) Castration removes the organs (the ovaries) which ordinarily inactivate some of the circulating gonadotrophin during their normal activity. After castration, gonadotrophins are no longer removed from the circulation and therefore increase; consequently, pituitary gonadotrophic content increases. (b) Castration removes the organs (the ovaries) which ordinarily secrete enough estrogens to sufficiently inhibit pituitary gonadotrophic secretion and content so that levels are kept low. After castration, inhibition of pituitary gonadotrophic secretion and content are removed; consequently, pituitary gonadotrophic content increases.

In neither the intact animal nor the castrate animal can the 2 hypotheses be tested separately. However, administering estrogens in physiological replacement amounts will prevent other postcastration changes but will not prevent the postcastration rise in gonadotrophin content of the pituitary.[1] This observation is not consonant with the "inhibition by estrogen" hypothesis; but, other than being a permissive, it adds no support to the "inactivation by the ovaries" hypothesis.

In order to test the "inactivation by the ovaries" hypothesis, the ovary must have access to the circulating gonadotrophins, so that if inactivation of gonadotrophins occurs, this can be detected. At the same time estrogens must be prevented from reaching the pituitary, enabling its potency to rise in the event that this is the correct explanation. These circumstances are met by auto-transplantation of both ovaries to the spleen. Thus estrogens are elaborated directly into the portal circulation and are immediately inactivated by the liver. Thus no estrogens reach the anterior pituitary, and its estrogenic environment is identical to that of a castrate. On the other hand, the ovaries remain available to the gonadotrophins in the systemic circulation and could inactivate them. Under these circumstances the gonadotrophic content of the pituitary should distinguish between the 2 hypotheses. For example, if "inhibition by estrogens" alone is responsible for controlling pituitary gonadotrophic activity, then the pituitary gonadotrophins should be as high as that seen following castration, since in both instances the possibility of "inhibition by estrogen" has been removed. On the other hand, if "inactivation by the ovaries" alone is responsible for controlling pituitary gonadotrophic activity, then the pituitary gonadotrophins should be as low as in the intact controls. If both mechanisms are in operation, a value between these 2 extremes should result.

In the *experimental group* (auto-transplantation of both ovaries to the spleen) (Fig. 3) no estrogens reach the pituitary gland. The lack of circulating estrogen is indicated in Table I and Fig. 3 by the fact that thymic and uterine weights and vaginal cells are at castrate levels.

The crucial question is: Did the pituitary glands of the spleen-transplant rats contain amounts of gonadotrophins comparable to the intact control level of 10.3 mg or comparable to the castrate control level of 92.4 mg? The pituitary gonadotrophin assays of 20.4 mg for the spleen-transplants clearly indicate that they are not comparable to the castrate controls (92.4 mg), but are comparable to the normal controls (10.3 mg). The gonadotrophin content is slightly but significantly elevated above normal.

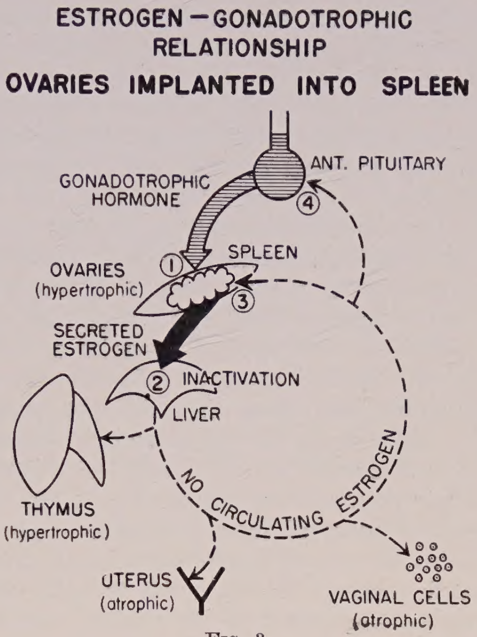

Fig. 3.

From this it can be concluded that the primary regulation of pituitary gonadotrophin content is the removal of active gonadotrophins from the circulation by the ovaries. It must further be concluded that inhibition by estrogen plays a definite but minor role in this control.

Histological examination of the pituitaries of each of the 3 groups warrants the identical assumption insofar as the pituitaries of the experimental group (ovaries transplanted to spleen) exhibited cells reminiscent of "castration cells." These, however, are not as well developed as for comparable castrates.

Are the changes observed in the experimental group not simply incidental to manipulation attending the transfer of ovaries to the spleen? Some operated rats developed collateral circulation from the spleen (containing ovaries) to the systemic circulation by way of adhesions. These served as unwitting but admirable controls. The ovaries in their new site were capable of elaborating estrogen in at least normal quantities as judged by the following findings: vaginal smears observed daily exhibited the same cycles as intact controls, thymus weights were identical to intact controls, and uterine development varied according to the stage of the cycle as in intact controls. It was somewhat puzzling therefore to find pituitary gonadotrophins slightly elevated above that of intact controls (28.8 mg). A possible explanation is that since the ovaries at first undergo partial atrophy following transplantation, and since they did not regain their preoperative size, they may have utilized less gonadotrophins. The eventual content of gonadotrophin in the pituitary seems to be a resultant of these 2 factors. This is further illustrated by administration of graduated amounts of α-estradiol to rats with ovaries transplanted to the spleen. Administration of large daily doses (5.0, 1.0 and 0.5 μg) for 34 days, from 6-18 days after operation to autopsy (Table I), caused total suppression of pituitary gonadotrophins. This was accompanied by complete ovarian atrophy for the 5.0 μg group. Had ovarian-utilization been the only mechanism in operation, the pituitary content would have been elevated to castrate levels. In contrast when smaller doses (0.25 and 0.05 μg) were administered, physiological levels of estrogen were approximated as judged by vaginal cells, uterine development and thymus weights. However, these dosages failed to permit ovarian growth (21.5 mg) to such an extent that they neither reached the spleen-transplant level of 149.5 mg nor their own operative control weight of 48 mg. Had inhibition by estrogen been the only mechanism in operation, the pituitary gonadotrophin content should have been as low as for intact controls (10.3 mg) instead of elevated to 22.3 mg. The elevation can be ascribed to failure of the smaller than normal ovaries to utilize the same amount of gonadotrophin as larger, normal ovaries would.

Summary. By autotransplantation of both ovaries into the spleen of mature female rats, the ovary continues to be bathed by pituitary gonadotrophins but the pituitary is no longer bathed by estrogens because in essence the liver has been inserted between the ovaries and the pituitary and has inactivated the estrogens.

The pituitaries of such rats were assayed for their gonadotrophic content. They more nearly resembled (20.4) that of intact controls (10.3), than that of castrated controls (92.4).

From this it was concluded that (1) ovaries normally inactivate gonadotrophins and that the rise of gonadotrophins seen following castration is due to failure of such inactivation to take place. (2) Large and unphysiological doses of estrogen are potent inhibitors of pituitary gonadotrophic potency. (3) Physiological amounts of estrogen in the circulation exert very little inhibitory action upon the pituitary.

15894

Regulation of Ovarian Growth: Inhibition by Estrogen or Stimulation by Gonadotrophins?

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In the preceding communication, it was demonstrated that the level of circulating gonadotrophic hormones fluctuates with the degree of ovarian activity. When ovaries are inactive or absent, they rise; when active, they fall. Perhaps ovarian growth and secretion are not solely regulated by the amounts of gonadotrophin present, but also by some other means. The results of estrogen administration to unilaterally castrated rats led

Heller, Heller and Sevringhaus[1] to suggest that the regulation of ovarian activity was the blood level of circulating estrogen. Thus a rise in blood level of estrogen could cause ovarian inhibition. Conversely a fall in blood level would remove inhibition and thereby stimulate the ovary.

Data are presented in this communication to indicate that estrogens secreted by the ovary act to inhibit growth of the ovary.

The concept that the circulating blood level of hormones secreted by a target-organ tends to regulate the production of hormone by that target organ is not without precedent. The analogous relationship between circulating thyroid-hormone and the production of thyroid-hormone was presented by Galli-Mainini.[2]

Control of ovarian growth and secretion in a normal intact animal may be by either (or both) of 2 mechanisms: (a) Stimulation of growth by pituitary gonadotrophin secretion (at position 1 in Fig. 1, preceding paper) and (b) active suppression of growth by estrogenic inhibition (at position 3 in Fig. 1, preceding paper).

To study this problem, a preparation must be devised which will separate these 2 possibilities. By autotransplanting the ovaries to the spleen, the ovaries remain under the influence of pituitary gonadotrophin stimulation; however, the estrogen secreted by the ovaries in the spleen is carried by the portal vein to the liver and inactivated, thus removing the ovaries from the effects of circulating estrogen. This is confirmed by the castrate appearance of the thymus, the uterus and the vagina of the experimental animals.

Materials and methods are described in the preceding paper.

Results are listed in Table I of the preceding paper.

Discussion. When ovaries were transplanted to the spleen, a marked hypertrophy occurred (normal, 49.9 mg, transplant, 149.5 mg). This could be due to increased production of pituitary gonadotrophin or to decrease in circulating estrogen reaching the ovaries. Assay of gonadotrophin content of the anterior pituitary glands of the transplant animals revealed relatively normal gonadotrophin levels (normal 10.3 mg, transplant, 20.4 mg) militating against the possibility of increased gonadotrophin stimulation. The hypothesis of decrease in circulating estrogens (removal of inhibition by estrogen) is favored by the 300% increase in ovarian weights noted in the autotransplants to the spleen. It is unlikely that the slight rise in pituitary gonadotrophic content accounts for the increase in ovarian weight, since the adhesion control rats developed similar gonadotrophic potency without similar ovarian growth. The inhibition of ovarian growth in the autotransplant rats with vascular adhesions to the systemic circuit must have been due to the presence of circulating estrogen in the blood.

To confirm this observation, two sets of animals were injected subcutaneously daily for 34 days with estradiol-benzoate. Group I received from 0.5 μg to 5.0 μg per day. These doses were greater than normally required by the animals, judged by the decrease in thymus weight from 154 mg to 132 mg, and the estrus condition of the uterus. Ovarian weights were markedly suppressed by the injections. The average at operation was 62.3 mg and after injection (at autopsy) 14.9 mg. Suppression of pituitary gonadotrophic potency to below normal was noted.

A second group was injected with 0.05 μg to 0.25 μg of estradiol benzoate daily. This lower dosage was more physiological, as judged by the normal weights and appearance of the thymus and uterus. However, not only was the 300% increase in ovarian weight prevented but actual atrophy occurred. The ovarian weights fell from 48.0 mg at operation to 21.5 mg at autopsy. This suppression in ovarian weight is not likely due to estrogen inhibition via the pituitary gland but due to direct inhibition of the ovary, since pituitary potency did not drop. In fact, as could be expected from the decrease in ovarian activity, the pituitary potency actually rose above normal during the period of estrogen administration.

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[1] Heller, C. G., Heller, E. J., and Sevringhaus, E. L., *Endocrin.*, 1942, **30**, 309.

[2] Galli-Mainini, C., *Endocrin.*, 1941, **29**, 674.

Whereas the presence of circulating pituitary gonadotrophin is the *sine qua non* of ovarian stimulation, in the presence of adequate amounts ovarian activity seems to largely be regulated by the level of circulating estrogens present.

Summary. Both ovaries of mature female rats were autotransplanted into the spleen, allowing the ovaries to be stimulated by pituitary gonadotrophins, but denying them the presence of circulating estrogen by interposing the liver between the ovaries and the systemic circulation.

The ovaries of these animals increased 3-fold in weight after 30-57 days transplantation (149.5 mg) over their weight at the time of operation (49.0 mg).

Two groups of experimental animals were injected with estradiol benzoate. One group received amounts exceeding physiological requirements and the other amounts approximately meeting physiological requirements. In both groups, the ovarian weights showed a marked decrease below the weight at operation.

It is concluded that ovarian growth is inhibited by the presence of circulating estrogens.

15895

Bodies Suggesting Exoerythrocytic Forms of *Plasmodium vivax* in Tissue Culture.*

I. N. DUBIN. (Introduced by Douglas H. Sprunt.)

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The demonstration of exoerythrocytic forms of avian malarial parasites[1] has stimulated a search for similar bodies in mammalian malaria. A few authors have described structures which they believed to be exoerythrocytic forms of human malarial parasites. The subject has been reviewed by Porter and Huff[2] and by Brug.[3] Brug[3] himself presented figures of bodies seen in smears of the lungs of a patient dying in the acute stage of *Plasmodium vivax* infection which he thought to be exoerythrocytic forms. Recently Raffaele[4] has reiterated the claim that he has seen such forms in all 3 types of human malaria.

To date, demonstration of exoerythrocytic forms has depended largely upon examination of smears and sections of tissues. Recently Hawking[5,6] has shown that the exoerythrocytic forms of avian malarial parasites are readily grown in tissue cultures of infected tissues. This has been confirmed for *P. gallinaceum* by Haas,[7] Zuckerman[8] and by the present author.[9] Since methods of tissue culture allow for multiplication of the histotropic forms of the parasites, this technic might prove superior to the use of smears or sections in the search for mammalian exoerythrocytic forms. Accordingly we have made tissue cultures of bone marrow from humans infected with sporozoites of *P. vivax* or *P. falciparum* both before and after parasites

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* This work was supported in part by a grant-in-aid from the Tennessee Valley Authority.

[1] Huff, C. G., and Bloom, W., *J. Infect. Dis.*, 1935, **57**, 315.

[2] Porter, R. J., and Huff, C. G., *Am. J. Trop. Med.*, 1940, **20**, 869.

[3] Brug, S. L., *Nederl. tijdschr. v. geneesk*, 1941, **84**, 2745.

[4] Raffaele, G., *Trop. Dis. Bull.*, 1946, **43**, 1016 (abstract).

[5] Hawking, F., *Trans. Roy. Soc. Trop. Med. and Hyg.*, 1945, **39**, 245.

[6] Hawking, F., *Trans. Roy. Soc. Trop. Med. and Hyg.*, 1946, **40**, 183.

[7] Haas, V. H., personal communication, 1946.

[8] Zuckerman, A., *J. Infect. Dis.*, 1946, **79**, 1.

[9] Dubin, I. N., in preparation.

appeared in the blood.[†]

As a preliminary measure the technic was tested by cultivating the exoerythrocytic forms of *P. gallinaceum* from infected chick embryos.[‡] These forms grew readily from spleen and liver as well as from brain and lung.[9] Hawking,[5] working with 10-day-old chicks, was unable to grow the parasites from the latter 2 tissues. Our difference in results is probably due to the greater ease with which embryonic tissues are cultivated as well as to the greater number of parasites in the embryonic tissues, as has been shown by Zuckerman.[8]

Methods and Materials. Sternal bone marrow punctures were made 4 to 6 days after the initial biting by infected mosquitoes, as well as 7 to 10 days after the development of parasitemia. The specimen was heparinized and centrifugalized. Tissue cultures were made from the fatty layer and from the buffy coat after it had been clotted with chick embryo extract. The cultures were made in bottomless Carrel flasks to which coverslips had been cemented.[10] The tissue was embedded in human plasma alone or in human plasma reinforced with chicken plasma. A drop of embryo extract was added to facilitate clotting. The fluid medium consisted of 25% human serum in Tyrode's fluid. The cultures were allowed to grow for 4 to 8 days at 37°-38°C. They were then fixed with formalin-Zenker fluid, stained with Maximow's stain, and mounted in clarite.[10]

Results. After negative results in 5 patients, 2 forms were found which bear resemblance to the exoerythrocytic forms of *P. gallinaceum.* These bodies were found in a 4-day-old culture of bone marrow taken 6 days after the patient had been bitten by mosquitoes infected with *P. vivax.* Five days after the sternal puncture was done parasites were demonstrated for the first time in the patient's blood.

Bodies found in tissue culture of human bone marrow from patient bitten by mosquitoes infected with *P. vivax.*

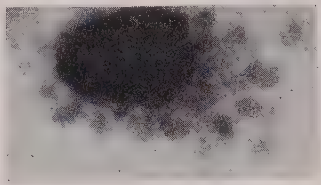

FIG. 1.
Photomicrograph of Form 1. Note the oval bodies surrounding the central nuclear mass. × 1600.

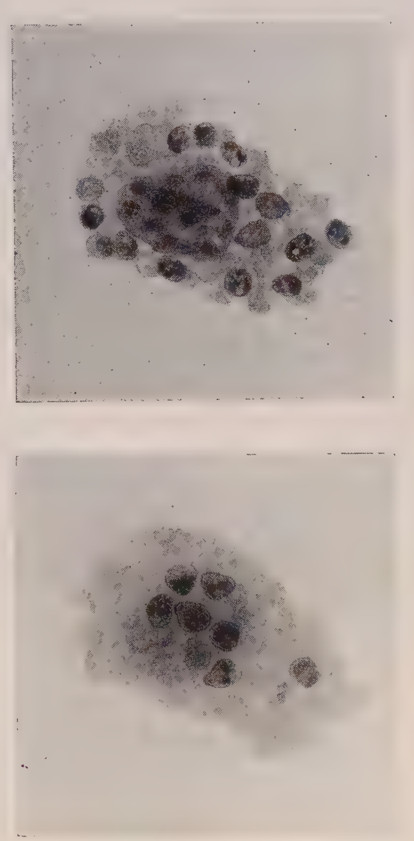

FIG. 2 AND 3.
Drawings of Form 1 at 2 different levels to show how nucleus is completely surrounded by oval bodies. × 1950.

The structures which we take to be exoerythrocytic forms of *P. vivax* are shown in Fig. 1 to 4. They were found in the immediate

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[†] The patients were neurosyphilitics receiving malarial treatment on the Neuropsychiatric Service of the Gailor Memorial Hospital, Memphis. The author is indebteed to Drs. T. S. Hill, Henry Packer, and Y. T. Wong for the use of the clinical material.

[‡] This material kindly supplied by Lt. Col. V. H. Haas, Office of Malarial Investigation, United States Public Health Service, Memphis.

[10] Zuckerman, A., *J. Inf. Dis.*, 1945, **77**, 28.

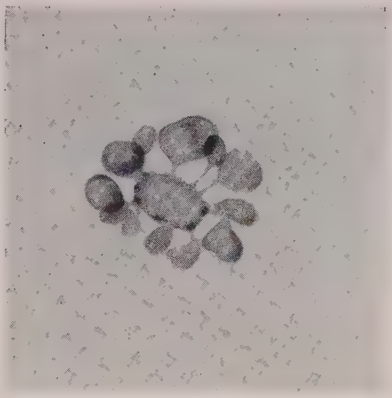

FIG. 4.
Drawing of Form 2. × 1800.

neighborhood of the implants. Form 1 (Fig. 1, 2, 3) shows a central nucleus surrounded by about 50 oval and round bodies. These small bodies have a sharp outline and an inner structure made up of vacuoles, wide pale-staining areas, and small dark granules. These bodies appear to be in the cytoplasm of a large macrophage. They resemble the larger merozoites described by Huff and Coulston.[11] Form 1 measured 21 by 12 μ.

Form 2 (Fig. 4) had a rosette appearance and showed a central core surrounded by radiating triangular or oval bodies. It measured 12 μ in diameter. It suggests a cluster of segmenters with a central core.

Discussion. These findings must be judged conservatively since (1) the forms found to date are so few, and (2) the criteria are purely morphological. The objection may be raised that in cultures of bone marrow degenerating cells are present which may be a source of error. The 2 bodies described above, however, are quite unlike the various forms taken by the degenerating cells. Moreover, their sharply-defined structures are suggestive of well-preserved animate bodies rather than of disintegrating cells. Furthermore, they resemble stages of exoerythrocytic forms in avian malaria.

Tissue culture studies are being continued in a search for additional forms in both human and simian malaria.

Addendum. After this article was submitted for publication there appeared a paper by Coulston and Huff[12] describing the exoerythrocytic forms of *Plasmodium relictum.* In the colored plates accompanying their paper are depicted forms which are similar to the 2 structures seen in the human bone-marrow. The oval bodies of the human Form 1 closely resemble the merozoites of *P. relictum* in Figs. 4, 7 and 13, while Form 2 is remarkably like the segmenter with a central core shown in Fig. 11.

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[11] Huff, C. G., and Coulston, F., *J. Infect. Dis.*, 1944, **75**, 231.

[12] Coulston, F., and Huff, C. G., *J. Infect. Dis.*, 1947, **80**, 209.

15896

Tuberculostatic Action of Two Derivatives of the Alicyclic Compound, 3-n-Amyl-Cyclopentane-Carboxylic Acid.

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In an earlier publication it was shown that the sodium salts of certain synthetic alicyclic acids had tuberculostatic action.[1] Among the compounds tested sodium 3-n-amyl-cyclopentane-carboxylate (referred to as compound "C") was found to be among the most effective, 10 mg % being sufficient to give complete inhibition of growth of the tubercle bacillus (Strain A27) in Kirchner's medium. When the tuberculostatic action of this compound was tested by the chick chorio-allantoic technic,[2] it was found that 0.5 mg of the compound in a suspension containing

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[1] Emmart, E. W., *Am. Rev. Tuberc.*, 1946, **53**, 83.

[2] Emmart, E. W., and Smith, M. I., *Am. Rev. Tuberc.*, 1943, **47**, 426.

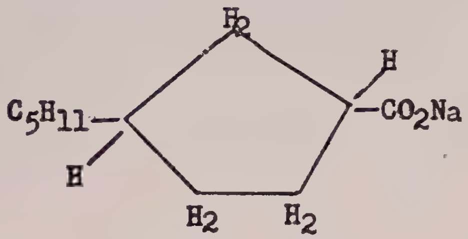
1 mg of tubercle bacilli, in doses of 1/5 cc, reduced the number of tubercles per membrane by 35%. This compound was therefore considered of sufficient interest to be used for further synthesis in the preparation of derivatives of possibly lower toxicity and higher tuberculostatic action.

Two new compounds have been prepared,* the mono-ester ("V") and the di-ester ("W") derived from sodium β-glycerophosphate and 3-n-amyl-cyclopentane-carboxylic acid.

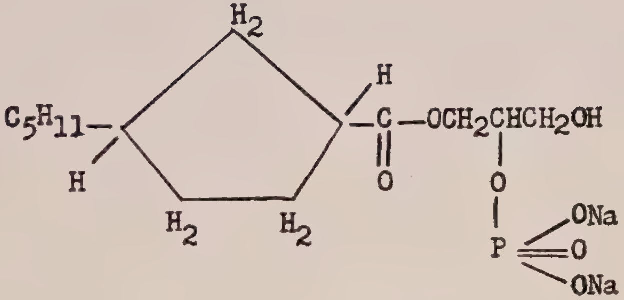
The 3 compounds referred to have the following formulae:

(Parent substance)

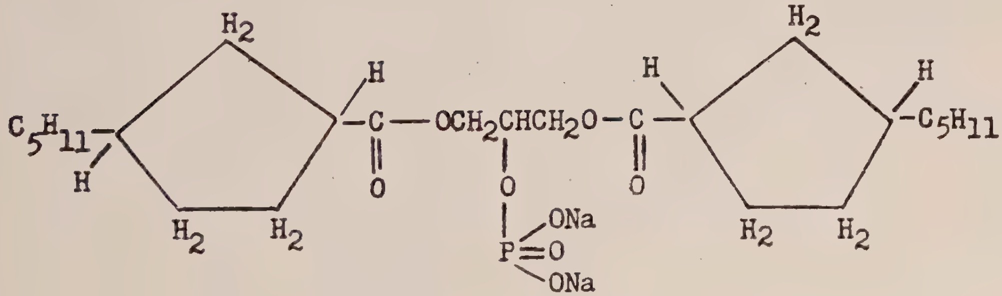
Sodium 3-n-amyl-cyclopentane-carboxylate ("C")



(Derivatives)
Mono-ester ("V")



Di-ester ("W")



Tuberculostatic action of the compounds "V" and "W" in Kirchner's medium.

In testing the tuberculostatic action of these 2 compounds dilutions of 1.2, 2.5, 5.0, 10.0 and 20.0 mg % have been used in Kirchner's medium with tubercle bacilli of the A27 human strain. The growth of the pellicle was observed and evaluated from 0 to 4, 4 representing the completely covered

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* We are indebted to H. Posvic, Cornell University, for the preparation of all alicyclic compounds and their derivatives.

TABLE I.
Tuberculostatic Action of Compounds "V" and "W" *in Vitro.*

Drug	Conc., mg %	Days observed and growth of pellicle (0-4)						
		5	10	15	20	25	30	35
"V"	20	—	—	—	—	—	—	—
"W"	20	—	—	—	—	—	—	—
"V"	10	—	—	0.1	0.1	0.2	0.2	0.3
"W"	10	—	—	—	—	—	—	—
"V"	5	0.6	1.1	1.2	1.2	1.2	1.3	1.4
"W"	5	—	0.5	0.5	0.6	0.7	0.7	0.7
"V"	2.5	0.5	1.0	1.2	1.2	1.4	1.5	1.9
"W"	2.5	—	1.1	1.1	1.2	1.2	1.3	1.3
"V"	1.2	0.9	1.1	1.3	1.3	2.9	3.2	3.5
"W"	1.2	0.3	0.9	0.9	0.3	1.1	2.4	2.8
Control	0.0	1.1	1.3	2.1	3.9	3.9	3.9	4.0

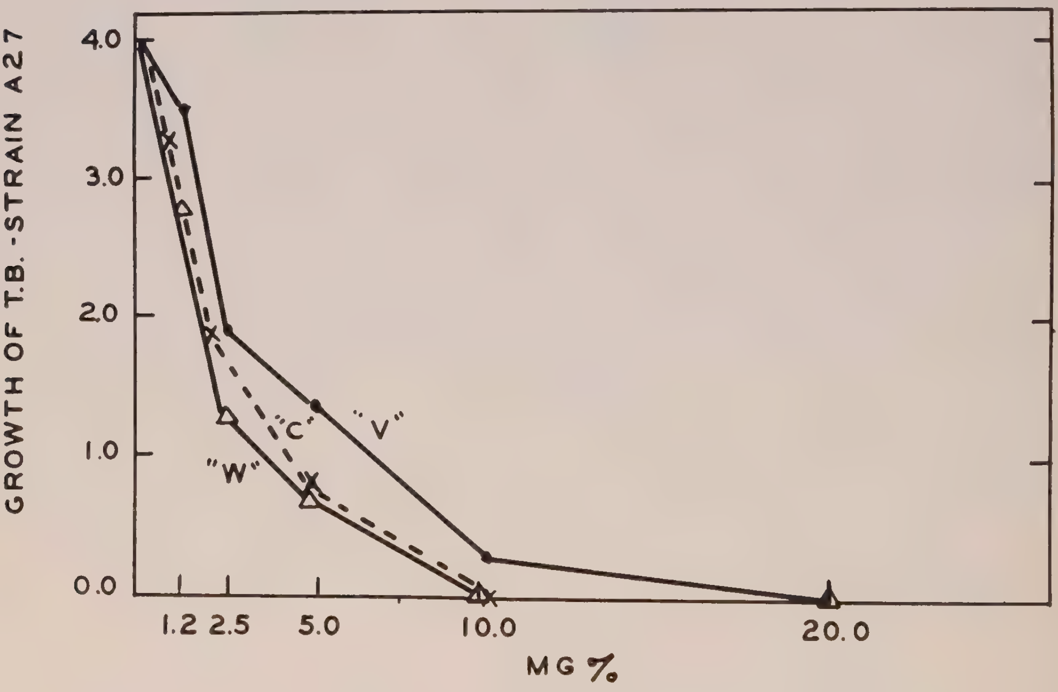

Chart 1.
Comparison of the tuberculostatic action of 3-n-amyl cyclopentane-carboxylic acid esters of β-glycerophosphate "V" and "W" with sodium 3-n-amyl-cyclopentane-carboxylate, "C," after 35 days in Kirchner's medium.

surface of the flask. Twenty mg % of compound "V" was found to give complete inhibition while 10 mg of "W" was sufficient to give similar results. Comparing the results with the other concentrations used, it was found in each dilution that compound "W," the di-ester, was more active than the mono-ester or compound "V" (Table I).

A comparison of the tuberculostatic action *in vitro* of the 2 derivatives of "V" and "W" with the parent substance sodium 3-n-amyl-cyclopentane-carboxylate indicates that there is no marked difference between the tuberculostatic action of compound "W" and "C" and both are more effective than "V" (Chart 1).

Toxicity of compound "V" and "W" as compared with the parent compound "C." In comparing the toxicity of "V" with that of "W" the effect of these compounds on the chick embryo was studied in doses of 0.6, 1.2, and 5.0 mg in 4 different experi-

TABLE II.
Toxicity of Compounds "W," "V," and "C" for Chick Embryos.

Drug	Dose, mg	No. of membranes inoculated	No. of embryos survived	% survival	% survival in relation to controls
"W"	5.0	20	0	0	
	2.5	60	9	15	35
	1.2	60	18	30	71
	0.6	20	9	45	107
	0.0 (Control)	80	34	42	
"V"	5.0	38	0	0	
	2.5	58	6	8	29
	1.2	91	22	24	88
	0.6	51	15	29	107
	0.0 (Control)	149	40	27	
"C"	4.0	30	1	3	6
	2.0	23	7	30	60
	1.0	22	8	36	72
	0.0 (Control)	48	24	50	

TABLE III.
Toxicity of the Compounds "C," "W," and "V" in White Mice.

Drug	Dose of drug in mg/20 g mouse	No. inoculated	No. survived	% mortality	Remarks
"C"	5.0	10	5	50	Survivors killed in 5 days
	7.5	11	4	64	" " " 3 "
	10.0	10	0	100	All died in 1½ hr
"W"	5.0	10	9	10	Survivors killed in 5 days
	7.5	10	2	80	" " " 3 "
	10.0	10	0	100	All died in 3½ hr
"V"	5.0	10	9	10	Survivors killed in 5 days
	7.5	10	4	60	" " " 3 "
	10.0	10	0	100	All died in 3 hr

ments. When the data were summarized it was found that 5 mg of both compounds "V" and "W" killed all the embryos while 0.6 mg had no effect upon the per cent of embryos which survived in the control and experimental groups (Table II). Since the survival rate in the control groups varied considerably with each experiment the survival rate of each of the experimental groups was calculated on a percentage basis in comparison with the corresponding control, a value of 100 being assigned to the latter in every case. Due to its low solubility compound "C" was used in concentrations of 4.0, 2.0, and 1.0 mg. The resulting data indicate that with chick embryos no appreciable difference in toxicity of "V" and "W" can be detected (Table II).

The toxicity of the 3 drugs was also studied in white mice (N.I.H. regular strain). The compounds were injected intraperitoneally in doses of 5.0, 7.5 and 10.0 mg per 20 g body weight. Ten mice were used for each dose. Ten mg of each compound was sufficient to kill all mice in less than 4 hours, 7.5 mg of "C" permitted 36% survival for 3 days, "V"—40% and "W"—20%. Five mg of "C" gave 50% survival in 5 days, while "W" and "V" permitted 90% survival in 5 days. All 3 drugs had a narcotic effect after intraperitoneal injection. Ten mg was sufficient to produce complete narcosis in 5 minutes. Five mg affected the mice within 5 minutes but the majority recovered and appeared normal in behavior. However, a few died within 24 hours after injection.

Autopsies on the remaining animals which were sacrificed either 3 or 5 days after injec-

TABLE IV.

Tuberculostatic Action of 3-n-Amyl-Cyclopentane-Carboxylic Acid Di-ester of Sodium β-Glycerophosphate ("W") and 3-n-Amyl-Cyclopentane-Carboxylic Acid Mono-ester of Sodium β-Glycerophosphate ("V") on the Chorio-allantois.

	Dose, mg	No. of membranes inoculated	No. of embryos survived	% survival	Avg tubercles per membrane (T.B. Strain H37Rv)	Chemotherapeutic activity*
"W"	1.25	24	4	16	1.0	8.8
	0.5	58	16	27	0.9	
	0.0 (Control)	77	32	41	8.0	
"V"	1.25	30	3	10	2.0	4.6
	0.5	60	7	11	0.7	
	0.0 (Control)	60	18	30	3.2	

* Chemotherapeutic activity is the ratio obtained by dividing the average number of tubercles in the control group by that of the treated groups.

tion showed that all 3 drugs in doses of 5.0 mg produced ascites. Hemorrhages were present at the site of injection but these were less marked with "V" than with compounds "W" and "C." There appears to be little difference between the toxicity of drugs "V" and "W," however, the more rapid killing action of compounds "C" indicates that both of the compounds "V" and "W" are less toxic than the parent substance (Table III).

Comparison of the tuberculostatic action of the compounds "V" and "W" on the chorio-allantoic membrane of the chick embryo. In order to test the inhibiting effect of these compounds on the development of tubercles in the chorio-allantoic membrane of the chick embryo suspensions were prepared of tubercle bacilli of H37-Rv strain containing 1.25 mg and 0.5 mg of each compound and 1 mg of tubercle bacilli per 1/5 cc of suspension. These were incubated 24 hours at 37.5° and inoculated on the chorio-allantoic membrane of the 9-day-old chick-embryo. Since small hemorrhages were frequently seen on the surface of the membranes when doses of 1.25 mg were used the chemotherapeutic activity of the 2 compounds was calculated on a basis of 0.5 mg per membrane, a dose sufficiently low as to have no visible toxic effect. The chemotherapeutic activity is expressed as the ratio obtained by dividing the average number of tubercles obtained in the control group by that of the treated group (Table IV). Since the chemotherapeutic activity in the group containing 0.5 mg of compound "W" was almost twice that of the group containing the "V" compound it appears that the di-ester is the more effective of the 2 drugs.

Summary. 1. Twenty mg % of 3-n-amyl-cyclopentane-carboxylic acid mono-ester of sodium β-glycerophosphate ("V") was found to give complete inhibition *in vitro*, while it required only 10 mg % of 3-n-amyl-cyclopentane-carboxylic acid di-ester of sodium β-glycerophosphate ("W") to give similar results.

2. The toxicity of 3-n-amyl-cyclopentane-carboxylic acid mono- and di-esters of sodium β-glycerophosphate for the chick embryo appears to be similar to that of the parent substance, sodium 3-n-amyl-cyclopentane-carboxylate. The lethal dose for chick embryos for compounds "V" and "W" is 5.0 mg. The less soluble parent compound "C" in concentrations of 4.0 mg permitted only 3% survival.

In white mice all 3 compounds had a narcotic effect and produced hemorrhages at the site of injection but the hemorrhage was less marked when compound "V" was used. Compound "C" in doses of 10 mg per 20 g mouse had a more rapid killing effect than either "V" or "W." Five mg of "C" gave 50% mortality, while "V" and "W" in the same dose gave only 10%.

3. When the tuberculostatic effect of "V" and "W" was tested on the chorio-allantoic membrane of the chick embryo the chemotherapeutic effectiveness of "W," the di-ester, was twice as great as the mono-ester "V."

15897

Effects of Sodium Nitrite and Certain Organic "Nitrates" on Blood Pressures of the Cat.*

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Herrman *et al.*[1] reported that in the etherized dog mannityl hexanitrate produced a greater lowering of carotid blood pressure than glyceryl trinitrate. Krantz *et al.*[2] concluded that isomannide dinitrate caused a more prolonged lowering of the carotid blood pressure of the etherized dog than glyceryl trinitrate, erythrityl tetranitrate or mannityl hexanitrate. In both experiments cited above the drugs were given by intravenous injection. In the paper by Krantz *et al.* there appear to have been no direct comparisons of isomannide dinitrate and glyceryl trinitrate or mannityl hexanitrate, the conclusions about the latter drugs being drawn from the literature. In neither paper is there data to allow one to assess the variability of the experimental results and the validity of the authors' conclusions.

The present experiment was undertaken to examine the effects of sodium nitrite and a number of organic "nitrates" on the carotid and right atrial pressures in such a way as to yield comparable results. The substances used were sodium nitrite ($NaNO_2$), isomannide dinitrate (I2N), glyceryl trinitrate (G3N), erythrityl tetranitrate (E4N) and mannityl hexanitrate (M6N).

Experimental. Cats were anesthetised by intraperitoneal injection of sodium pentobarbital, the doses being calculated from a curve similar to that of Bazett and Erb.[3] They were prepared then for recording respiration by lateral pressure, carotid blood pressure and right atrial pressure. The blood of the animal was rendered noncoagulating by intravenous injection of 1 ml/kg of an 8% solution of chlorazol fast pink. Drug solutions were washed into a saphenous vein with saline.

The compounds studied, except $NaNO_2$, were dissolved in a solvent consisting of 80 g of urethane in sufficient 95% alcohol to make 100 ml of solution. $NaNO_2$ was dissolved in a similar solvent containing water in place of alcohol. The aqueous and alcoholic solvents had identical effects. Solutions of the drugs were made to contain 4 mg of drug per ml, a dose of 0.25 ml of solution per kg of cat being used in all experiments.

The various solutions, including the solvent, were injected serially into each cat, at least one-half hour elapsing between successive injections. The order of administration of the 6 solutions was varied in a cyclic manner through the series of 12 cats, so that there were 2 such cycles.

Table I shows the actions of the solvent and the 5 drug solutions on the mean carotid pressure and the right atrial pressure. It is evident that while the mean effects on carotid pressure show differences which agree in some respects with those reported previously in the etherized dog,[1,2] the variations in the present experiment were so great as to force the conclusion that equal doses of the 5 drugs used here have roughly identical effects on mean carotid pressure. There is no evidence that the same thing is not true of the previous experiments.

When the atrial pressures are considered, it is apparent that $NaNO_2$ and E4N differ from the other drugs and from each other. Both of these drugs produced a lowering of

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* Supported by a grant from the R. J. Strasenburgh Company, Rochester, N.Y.

[1] Herrman, R. F., Leake, C. D., Loevenhart, A. S., and Muehlberger, C. W., *J. Pharm. Exp. Therap.*, 1926, **27**, 259P.

[2] Krantz, J. C., Jr., Carr, C. J., Forman, S. E., and Ellis, F. W., *J. Pharm. Exp. Therap.*, 1939, **67**, 191.

[3] Bazett, H. C., and Erb, W. H., *J. Pharm. Exp. Therap.*, 1933, **49**, 352.

TABLE I.
Effect of Sodium Nitrite and Organic Nitrates on the Mean Carotid Pressure and Right Atrial Pressure of the Cat Anesthetized with Nembutal.

Drug	Mean carotid pressure				Right atrial pressure			
	Mean*	Maximal† drug effect	Mins. to max. effect	Mins. to recovery	Mean‡	Maximal§ drug effect	Mins. to max. effect	Drug effect‖ at 8 mins.
Solvent	141	— 13 ± 35	3.7	7.7	—11.2	+1.0 ± 1.4	0.4	+0.1 ± 0.1
M6N	134	—81 ± 36	0.9	6.1	—16.2	+1.2 ± 0.9	1.7	+1.1 ± 0.7
E4N	129	— 65 ± 23	0.7	8.6	—13.3	+2.2 ± 1.8	1.2	—2.0 ± 1.6
G3N	153	— 92 ± 29	0.8	5.5	—15.5	+4.5 ± 1.2	1.6	+2.5 ± 0.8
I2N	135	—59 ± 26	1.0	6.0	—13.5	+2.5 ± 2.1	1.6	+0.3 ± 0.3
$NaNO_2$	141	—46 ± 35	0.5	8.0	—11.5	—3.7 ± 1.4	0.4	—1.5 ± 0.7

* Mm of mercury pressure.
† Mean maximal change in mm of mercury ± standard deviation of mean.
‡ Mm of water pressure.
§ Mean maximal change in mm of water ± standard deviation of mean.
‖ Mean change in mm of water ± standard deviation of mean.

right atrial pressure at a time (8 minutes after the injection) when the other substances caused or permitted increases in this pressure. $NaNO_2$ differs from E4N in that it produced an immediate fall in atrial pressure while the fall after injection of E4N was a delayed reaction.

The long period for which the atrial pressure remained lowered after the administration of $NaNO_2$ probably indicates that in the cat the principal prolonged action of this drug is on the venous side of the circulation, as has been found to be the case in man by Weiss *et al.*[4] The eventual fall of atrial pressure after injection of E4N to about the level obtained by giving $NaNO_2$ means possibly that the former drug has a prolonged but weak dilating action on the venous circulation, which is masked at first by the increased return through dilated arterioles.

The effect of E4N on the atrial pressure is of especial interest in that it suggests that E4N is the only one of the 4 organic "nitrates" used here which has a vasodilator action as the result of a mechanism similar to that of $NaNO_2$. Various investigators have found evidences[5–7] that I2N, G3N, E4N and M6N do not act in the body by the release of nitrite ion as postulated by Herrman *et al.*[1] The partial similarity between the actions of E4N and $NaNO_2$ on the atrial pressure certainly does not prove that E4N releases nitrite ion but it does mirror some difference in behavior in the body between E4N and the other organic "nitrates." This difference might involve the slow release of nitrite ions by E4N at such a rate as to produce no significant methemoglobinemia or elevation of the level of nitrite ion in the general circulation.

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[4] Weiss, S., Wilkins, R. W., and Haynes, F. W., *J. Clin. Invest.*, 1937, **16**, 73; Wilkins, R. W., Haynes, F. W., and Weiss, S., *J. Clin. Invest.*, 1937, **16**, 85.

[5] Crandall, L. A., Jr., Leake, C. D., Loevenhart, A. S., and Muehlberger, C. W., *J. Pharm. Exp. Therap.*, 1931, **41**, 103.

[6] Krantz, J. C., Jr., Carr, C. J., Forman, S. E., and Cone, N., *J. Pharm. Exp. Therap.*, 1940, **70**, 323.

[7] Rath, M., and Krantz, J. C., Jr., *J. Pharm. Exp. Therap.*, 1942, **76**, 33.

Summary. Sodium nitrite ($NaNO_2$), isomannide dinitrate (I2N), glyceryl trinitrate (G3N), erythrityl tetranitrate (E4N) and mannityl hexanitrate (M6N) injected intravenously in doses of 1 mg/kg into the cat anesthetized with pentobarbital had effects on the mean carotid blood pressure which did not differ significantly in magnitude and duration.

$NaNO_2$ appeared to have an immediate and powerful dilating action on the venous system while E4N may have had a similar but weaker action. I2N, G3N and M6N appeared not to affect the venous system significantly.

Dr. J. C. Krantz, Jr., of the University of Maryland Medical School, kindly supplied the isomannide dinitrate.

15898

Influence of Protein-Binding on the Interpretation of Penicillin Activity *In vivo.**

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Introduction. In evaluation of the therapeutic use of penicillin and other antibacterial agents, considerable interest was centered on observations of the height and duration of the serum concentrations attained after administration to humans. Studies of this type have been of value in determining the dosage of penicillin, the frequency and route of administration, and the influence of various vehicles and adjuvants on absorption and excretion.

The availability of the individual purified penicillins, X, G, F, K and dihydro F, has stimulated renewed interest in this type of investigation. It has been reported that penicillin X affords higher and more prolonged concentrations in serum than an equal dose of penicillin G.[1,2] Likewise penicillin X has been found to be significantly more effective than penicillin G in the treatment of infections in experimental animals.[3,4]

Of particular interest have been the studies of penicillin K. *In vitro,* penicillin K is distinctly more active than penicillins X, G, F and dihydro F. In the treatment of infections in experimental animals, however, penicillin K has been found to be the least effective of these 5 penicillins.[3,4] It was observed simultaneously in 3 laboratories[5-7] that, following intramuscular injection, penicillin K apparently disappeared rapidly from the circulating blood. It was also observed that the total urinary excretion of penicillin K was low, and it was concluded that the drug was ineffective because of rapid destruction in the body.[5,6]

Eagle[8] has reported a possible explanation of the differences in pharmacologic behavior among the individual penicillins. In a study of penicillins X, G, F and K, he observed that all 4 penicillins were slowly inactivated by a thermostable component of serum. Penicillin X was the least susceptible of the 4 to this inactivation. In addition, he reported that penicillin K was rapidly inactivated by a thermolabile component of serum, and that the rate of this latter type of inactivation increased as the concentration of penicillin decreased. Eagle postulated that the apparent differences in the periods during which the individual penicillins were present in the circulating blood could be explained on the basis of the relative susceptibilities of these penicillins to inactivation by serum *in vitro*.

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* This investigation was conducted with a grant-in-aid from the National Institute of Health. The study was also aided in part by a grant from the Lederle Laboratories, Pearl River, N.Y.

[1] Ory, E. M., Meads, M., and Finland, M., *J. A. M. A.*, 1945, **129**, 157.

[2] Eagle, H., *J. Exp. Med.*, 1947, **85**, 163.

[3] Hobby, G. L., Burkhart, B., and Hyman, B., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 296.

[4] Eagle, H., *J. Exp. Med.*, 1947, **85**, 175.

[5] Eagle, H., and Musselman, A., *Science*, 1946, **103**, 618.

[6] Coghill, R. D., Osterberg, A. E., and Hazel, G. R., *Science*, 1946, **103**, 709.

[7] McDermott, W., and Tompsett, R., cited in *J. A. M. A.*, 1946, **131**, 271.

[8] Eagle, H., *J. Exp. Med.*, 1947, **85**, 141.

In contrast to previous observations on penicillin K, Richardson and his co-workers[9] have recently reported that from a given plasma concentration the rate of disappearance of penicillin K is the same as that of penicillin G. These investigators found in dogs that after the intravenous administration of penicillins G and K the penicillin K was localized in the liver in higher concentrations than penicillin G. They further reported that both penicillins appeared to be bound by plasma but that penicillin K was bound to a greater degree than penicillin G. Evidence was obtained that the low recovery (activity) of penicillin K in plasma was not due to destruction of the penicillin.

In a previous communication from this laboratory,[10] it was reported that the antibacterial activities of penicillins X, G, dihydro F and K were antagonized by serum and the albumin fraction of serum. The degrees of antagonism were quantitatively different for the individual penicillins. Among these 4 penicillins, the degrees of reduction in antibacterial activity caused by serum and by albumin were roughly in direct proportion to the degrees of binding to these substances demonstrable by dialysis. Penicillin X, which was bound 47%, lost 40 to 60% of its *in vitro* activity in the presence of serum or albumin. Penicillin K was bound approximately 90%, and when similarly tested lost 85 to 90% of its activity. The degrees of binding and the degrees of reduction in activity of penicillins G and dihydro F in serum were intermediate between the values for penicillins X and K. Moreover, the reduction in antibacterial activity was independent of any actual destruction of penicillin by the serum, and was the same in fresh serum as in serum heated at 56°C for 30 minutes.

These data indicate that the antibacterial activity of the individual penicillins is exerted only by the unbound portion of the drug. The observations afforded a possible explanation of the apparent discrepancies between the *in vitro* and *in vivo* activities of the 4 penicillins studied. When the *in vitro* testing was performed in the presence of serum or the albumin fraction of serum, the relative activities of penicillins X, G, dihydro F and K were the same *in vitro* as *in vivo*.

In addition it was reported that the phenomena observed interfere with the bioassay of penicillin in serum, and the interference is quantitatively different for each penicillin. The measurement of penicillin in serum by a tube dilution method depends on the determination in a standard control system of the sensitivity of a test organism to penicillin. From this value, the penicillin concentration in an unknown specimen is calculated according to the following formula:

$$\text{units per cc in unknown} = \frac{\text{sensitivity of test organism (u/cc)}}{\text{reciprocal of the highest dilution of unknown specimen which inhibits growth}}$$

For this calculation to be valid, *the sensitivity of the test organism must be constant throughout every dilution of the bioassay procedure.* It was observed, however, that the values for the sensitivity of 2 common test organisms — *Streptococcus hemolyticus* (C203MV) and *Staphylococcus aureus* (Oxford H)—vary in different concentrations of serum, an effect which is presumably due to binding of the penicillin to albumin. The differences in sensitivity were thus quantitatively different among the individual peni-

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[9] Richardson, A. P., Miller, I., Schumacher, C., Jambor, W., Pansy, F., and Lapedes, D., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 514.

[10] Tompsett, R., Shultz, S., and McDermott, W., *J. Bact.*, 1947, **53**, 581.

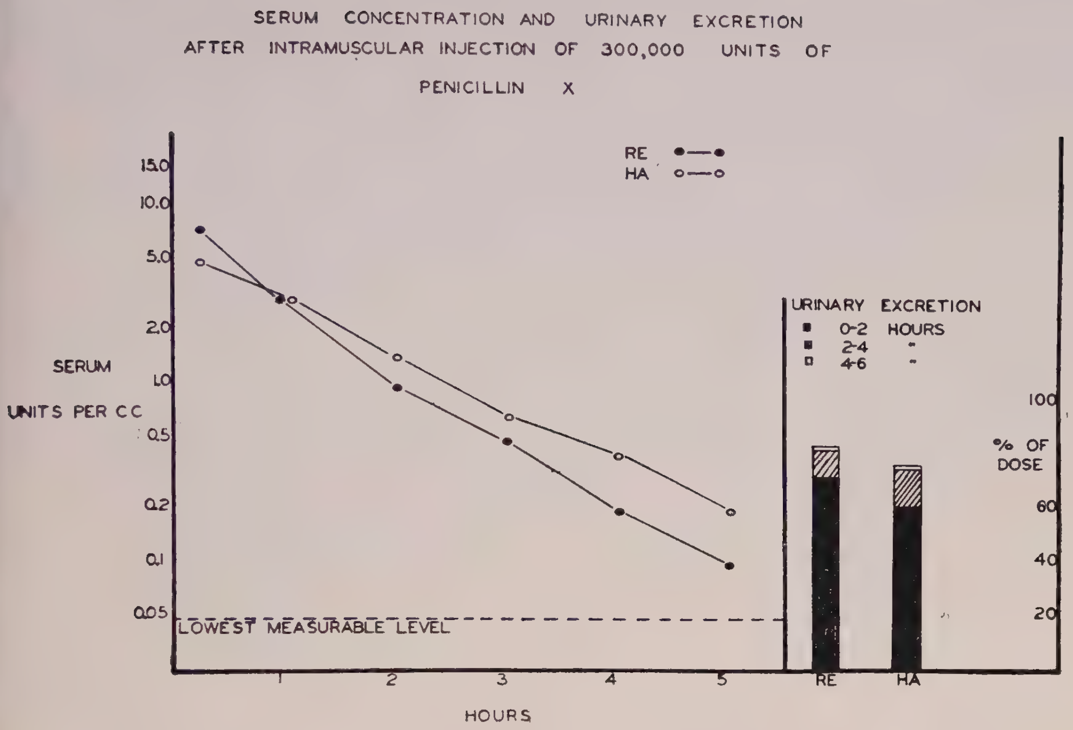

FIG. 1.

cillins and were most marked with penicillin K. A method of bioassay was developed in which the effect of serum was kept constant by maintaining a constant concentration in each tube through the addition of pooled serum. By this method, using *Streptococcus hemolyticus* C203MV as the test organism, the lowest concentration of the 4 penicillins measurable in serum varied from 0.05 unit per cc of penicillin X to 0.4 to 0.5 unit per cc of penicillin K.

These observations suggested that the findings of prolonged serum concentrations of penicillin X, and the rapid "disappearance" of penicillin K from the blood might be the result of failure to consider the effects of serum on the bioassay itself. Accordingly it seemed advisable to evaluate the absorption, excretion and comparative rates of disappearance in the light of the differences in the degrees to which they are bound by serum.

Experimental. Methods and Materials. Four crystalline penicillins[†] were used: (1) *p*-hydroxybenzylpenicillin (X), (2) benzylpenicillin (G), (3) *n*-amylpenicillin (dihydro F), and (4) *n*-heptylpenicillin (K).[‡]

The penicillins were administered intramuscularly to healthy adults in single doses dissolved in normal saline solution. Assays of penicillin in serum were performed by the method previously described.[10] The test organism was *Streptococcus hemolyticus* C203-MV. Measurements of penicillin in urine were performed by a similar tube dilution method. A few of the urine samples were

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[†] The penicillins G, dihydro F and K were supplied by Charles Pfizer and Co. The penicillin X was obtained from Lederle Laboratories.

[‡] The sample of penicillin K was a crystalline sodium salt which by the method of Craig and his associates[11] was shown to contain approximately 80% of n-heptylpenicillin, and 15% or more of other as yet unidentified antibiotics. Although this material did not consist entirely of n-heptylpenicillin the biologic and pharmacologic behavior appears to be sufficiently distinctive to warrant the use of the term K or "K-type" penicillin.

[11] Craig, L. C., Hogeboom, G. H., Carpenter, F. H., and duVigneaud, V., *J. Biol. Chem.*, in press.

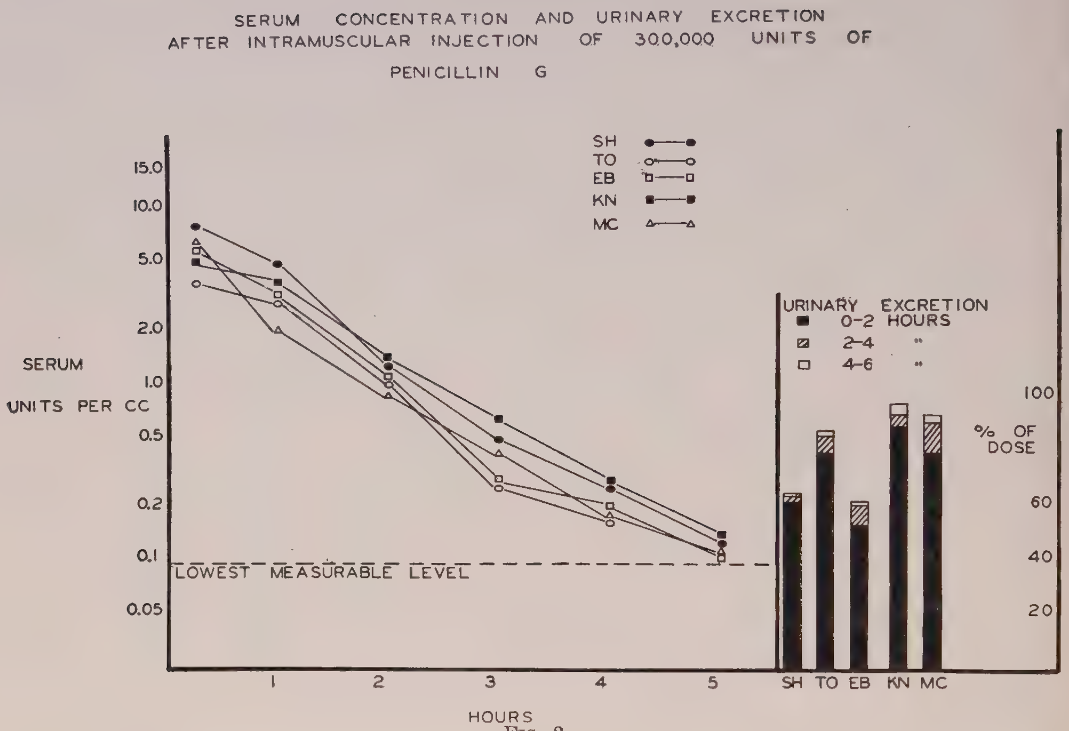
SERUM CONCENTRATION AND URINARY EXCRETION
AFTER INTRAMUSCULAR INJECTION OF 300,000 UNITS OF
PENICILLIN G
SH
TO
EB
KN
MC
SERUM
UNITS PER CC
15.0
10.0
5.0
2.0
1.0
0.5
0.2
0.1
0.05
LOWEST MEASURABLE LEVEL
1
2
3
4
5
HOURS
URINARY EXCRETION
0-2 HOURS
2-4 "
4-6 "
100
% OF DOSE
60
40
20
SH
TO
EB
KN
MC

FIG. 2.

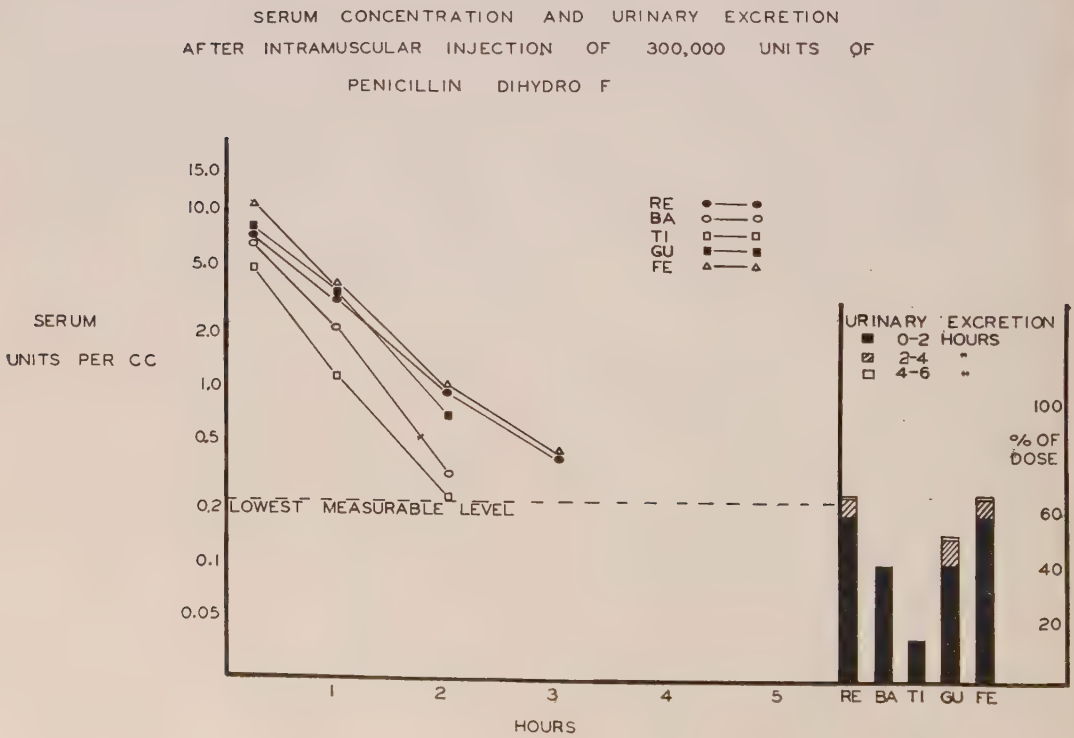
SERUM CONCENTRATION AND URINARY EXCRETION
AFTER INTRAMUSCULAR INJECTION OF 300,000 UNITS OF
PENICILLIN DIHYDRO F
RE
BA
TI
GU
FE
SERUM
UNITS PER CC
15.0
10.0
5.0
2.0
1.0
0.5
0.2
0.1
0.05
LOWEST MEASURABLE LEVEL
1
2
3
4
5
HOURS
URINARY EXCRETION
0-2 HOURS
2-4 "
4-6 "
100
% OF DOSE
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FIG. 3.

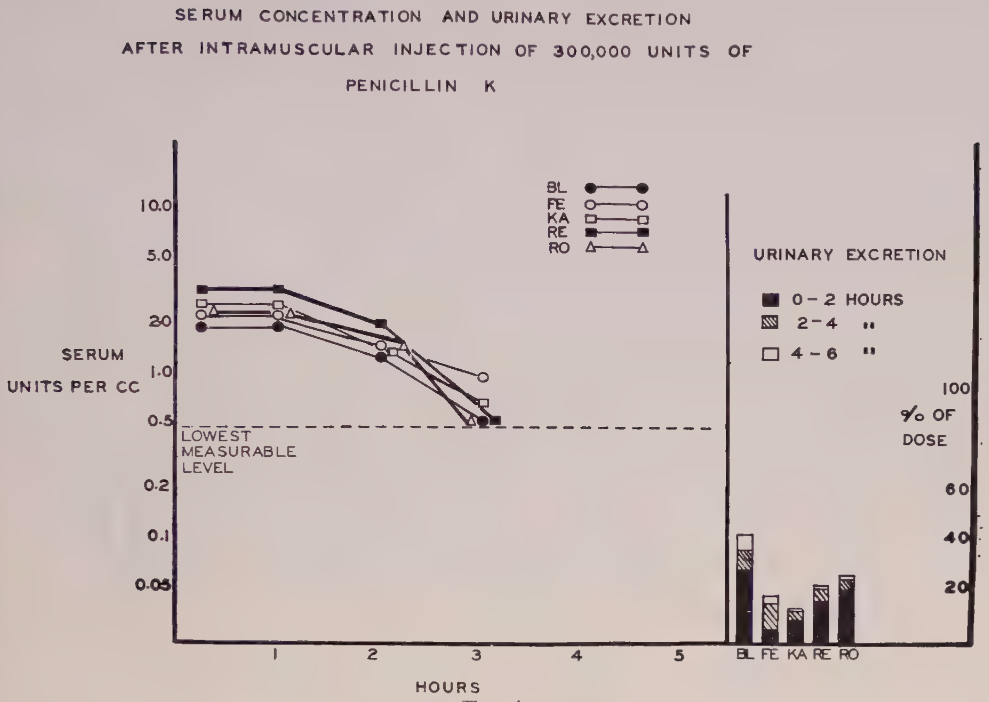

Fig. 4.

also assayed by the standard Oxford cup plate method.

Results. In Fig. 1 to 4 may be seen the serum concentrations of the 4 penicillins attained in the individual subjects after single doses of 300,000 units. The serum concentrations of penicillin X (Fig. 1) in the 2 subjects tested were highest at 15 minutes and fell at a regular rate throughout the test period. In both instances, penicillin X was present in concentrations well above the lowest measurable concentration at the end of the experiment. In Fig. 2 may be seen the values obtained in 5 subjects who received penicillin G. The results were essentially identical with those obtained with penicillin X. The range of values among the different subjects at each time period was small. At the end of 5 hours, the serum concentrations of penicillin G in 4 of the 5 subjects were still measurable.

In Fig. 3 are shown the results of a similar experiment with penicillin dihydro F. With this penicillin the effect of serum in the assay was more noticeable. When the concentration of serum was properly controlled the lowest measurable level of penicillin dihydro F was 0.25 unit per cc. In 3 of 5 subjects, the serum concentration had fallen below this level at the end of the 3rd hour, and in the other 2 at the end of the 4th hour.

In Fig. 4 may be seen the values obtained with penicillin K. The values in the individual subjects were remarkably uniform. Fifteen minutes after injection the levels were not so high as were observed with the other penicillins. One hour after administration, however, the penicillin concentrations were at the same level as had been present at 15 minutes. Moreover, at one hour, the height of the concentrations of penicillin K was essentially the same as was seen at the same interval after the administration of the other penicillins. It is evident that the rate of fall in serum concentrations of penicillin K was not rapid as compared with the other penicillins. A comparison of Fig. 4 with Fig. 1 and 2 discloses that in every instance the concentrations of penicillins X and G at the end of 4 hours were well below the lowest level at which penicillin K can be detected (0.5 unit per cc). Thus it is impossible to

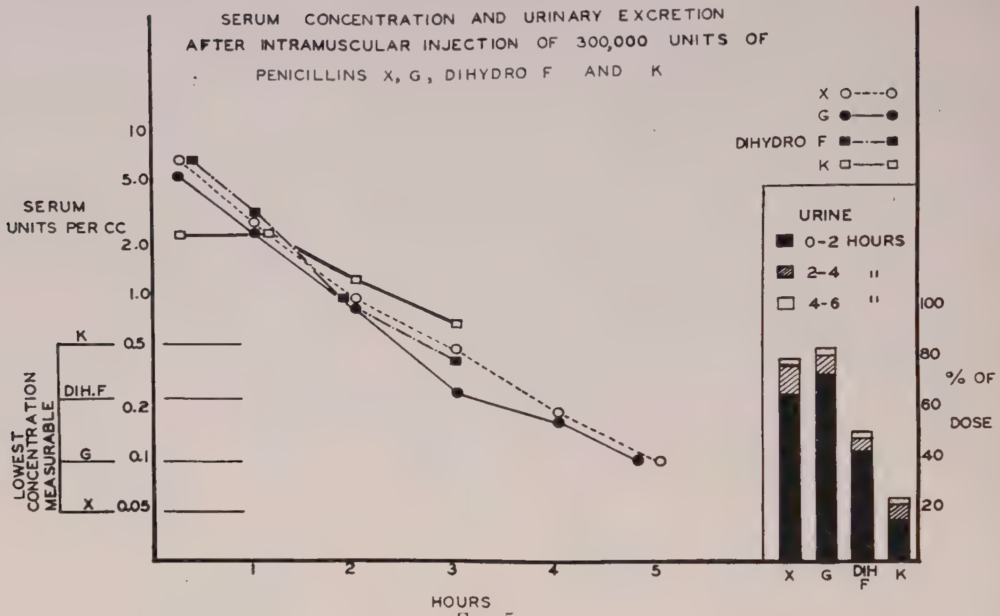

FIG. 5.

establish whether penicillin K was also present in the same concentrations as the other penicillins at the end of the 4th hour after injection. From the form of the curves, however, it is highly probable that the disappearance of penicillin K after the 3rd hour is only a reflection of the limitation of the bio-assay of penicillin K.

The similarity of the levels of the 4 penicillins may be illustrated by the results in a single subject. In Fig. 5 are presented the values obtained with the 4 penicillins in a single representative subject. *As may be seen during the first 3 hours, when all the penicillins were present in measurable concentrations, there was no difference among the 4, except for the lower peak of penicillin K at 15 minutes.* In this subject the concentration of penicillin K 3 hours after injection was actually slightly higher than was observed at the same interval after the administration of the other 3 penicillins. (The mean serum concentration of penicillin K in the 5 subjects 3 hours after injection was also higher than the mean values obtained with the other penicillins). Four or 5 hours after injection the concentrations of penicillins X and G although low were above the minimum detectable level for these particular penicillins.

The influence of the antagonistic action of serum on the interpretation of the values obtained with penicillin K may also be demonstrated by a comparison of 2 methods of assay. In Fig. 6 may be seen the serum concentrations of penicillin K in one of the subjects as measured simultaneously by 2 methods. The solid line represents the values obtained with the method described above. The broken line represents values obtained when the same sera were assayed by a standard dilution method in which no correction is made for the influence of serum in the individual dilutions of the assay. The latter curve is similar to those previously reported in studies of penicillin K.[5,6] As may be seen in Fig. 6, when high concentrations are present, the values obtained by the 2 methods are nearly the same. As the concentration in the serum falls, however, the difference becomes increasingly greater. The lower the actual concentration of penicillin becomes, the more of the unknown serum will be present in the tube in which the endpoint occurs. Therefore, as the concentration of penicillin decreases the error in this type of assay is magnified as a result of failure to consider that the antagonistic action of serum at the endpoint has been increased. Thus, failure to consider these opposing factors in this experiment with penicillin K caused the

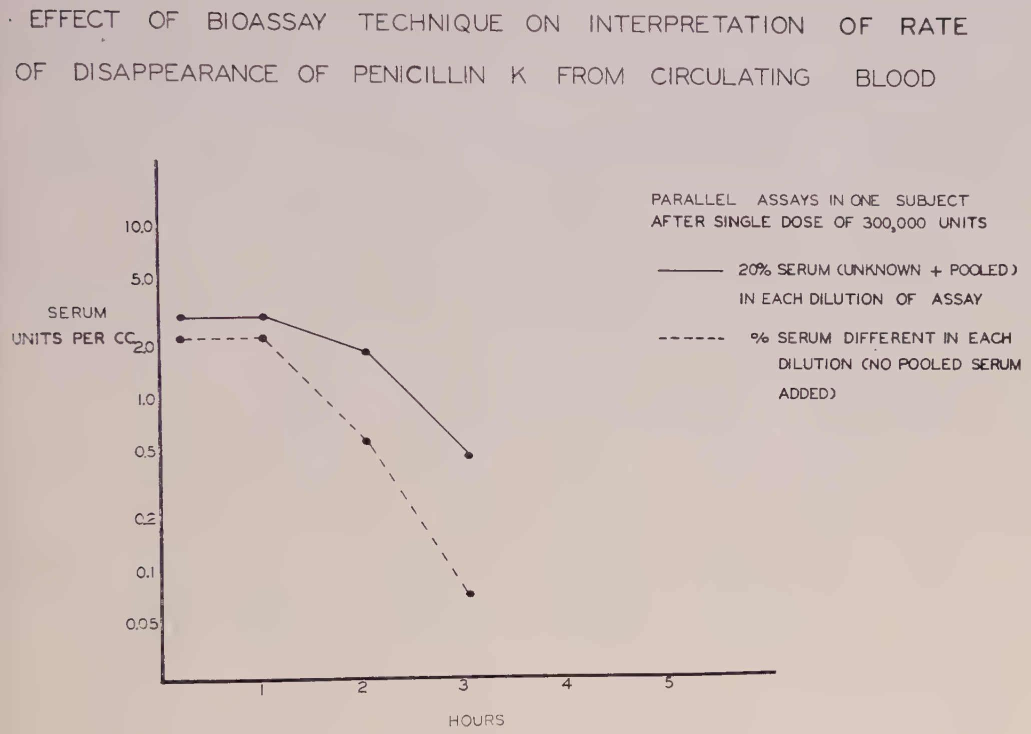

FIG. 6.

rate of fall in serum concentration to appear rapid, and *increasingly more rapid as the actual concentration became lower.*

Although the data which were obtained with penicillin X are too few to be conclusive, it is of interest to compare the values for serum concentrations of penicillins X and G as presented in Fig. 1 and 2. As may be seen there was no significant difference in the serum levels of these 2 penicillins during the course of the experiment. At the end of 5 hours, however, in 4 of the 5 subjects who received penicillin G, the serum concentrations were just above the lowest measurable level. If the experiment had been carried out for a longer period, it is likely that penicillin X could have been measured for a longer time than penicillin G.

Similar studies were carried out with doses of 20,000 units. The serum concentrations attained in the individual subjects are shown in Table I. The general distribution of the values was the same with all 4 penicillins. As in the previous experiments during the time in which all 4 penicillins were measurable, there was no great difference in concentration among the individual penicillins. After 30 to 60 minutes, with only one exception, the concentrations of penicillin X, G and dihydro F fell to levels which were well below the lowest level at which penicillin K can be measured.

In Fig. 1 to 4 are also presented the cumulative urinary excretions of the 4 penicillins for a 6-hour period after administration. The average excretion of penicillin X was 82%; penicillin G, 84.6%; penicillin dihydro F, 51.4%; and penicillin K, 24%. These results are in general agreement with those previously reported.[6,7] From other studies of substances bound to a high degree by plasma protein, it might be anticipated that the degree of binding of penicillin K would affect its rate of excretion in the urine. In Table II may be seen the 6-hour excretion of the 4 penicillins, expressed in terms of percentage of the total excreted in each 2-hour period. Despite considerable variation from one subject to another, it is apparent that the pattern of excretion of penicillin K is

TABLE I.
Serum Concentrations (Units per cc) of Penicillins X, G, Dihydro K and F After Intramuscular Injection of 20,000 Units.

Penicillin	Lowest measurable levels in serum controls units per cc	Minutes after administration				
		15	30	60	90	120
K	0.5	1.4	0.7	—	—	—
		0.5	—	—	—	—
		0.7	—	—	—	—
Dihydro F	0.25	0.5	0.415	—	—	—
		0.5	0.35	—	—	—
		0.5	0.25	—	—	—
		0.7	0.35	—	—	—
		0.7	0.35	—	—	—
G	0.15	0.6	0.42	0.2	—	—
		1.25	0.75	0.15	—	—
X	0.05	0.67	0.67	0.325	0.1	0.05
		1.5	1.0	0.5	0.25	0.14

TABLE II.
Time Relationships of the Urinary Excretion of Penicillins X, G, Dihydro F and K During a 6-Hour Period After Intramuscular Injection of 300,000 Units.

Penicillin	Subject	% of total 6-hr excretion recovered in each 2-hr period		
		0-2 hr	2-4 hr	4-6 hr
X	Re	86	12	2
	Ha	80.5	18	1.5
	Avg	83.2	15	1.75
G	Sh	96	2.8	1.2
	To	91.6	6.6	1.8
	Eb	84.7	12.1	3.2
	Ku	86	10.5	3.5
	Mc	85.5	12	2.5
	Avg	88.9	8.8	2.4
Dihydro F	Re	89.3	9.9	0.8
	Ba	99	0.4	0.6
	Ti	91	7.1	1.9
	Gu	80	18.0	2.0
	Fe	88	9.6	2.4
	Avg	89.4	9.0	1.5
K	Be	70	17	13
	Fe	32.7	52	15.3
	Ka	67.5	27.5	5
	Re	77	22	1
	Ro	76	16.6	7.4
	Avg	64.6	27	8.3

distinctly different from the other 3 penicillins, in that relatively high percentages of the total excretion occur after the first 2 hours. Moreover, the average renal clearance of penicillin K in the 5 subjects was less than half that of penicillins G and X.

Discussion. From the data presented it appears that consideration of the antagonistic action of serum, which presumably occurs as a result of protein-binding, materially alters the previous interpretations of the serum levels attained after intramuscular administration of the crystalline penicillins studied. The serum concentrations of penicillins X, G, dihydro F and K attained after intramuscular injection were remarkably uniform during the period when all 4 were measurable by a method which allows constant conditions of binding by serum protein. The 2 chief features previously reported for the individual penicillins, *e.g.*, higher and more prolonged serum concentrations afforded by penicillin X, and lower and poorly sustained concentrations by penicillin K, were not observed.

Of particular interest was the observation that in each of the 5 subjects who received 300,000 units of penicillin K, the serum concentrations at 60 minutes were precisely the same as had been present 15 minutes after administration. The explanation of this is not apparent. In view of other observations on penicillin K, and its similarity to the other penicillins, it is conceivable but unlikely that the sustained serum concentration during the first hour is the result of delayed absorption. The findings may reflect the fact observed by Richardson and his co-workers, that penicillin K is distributed in the body differently than the other penicillins. Alteration in renal excretion caused by protein-binding might also contribute to this phenomenon of a constant concentration during the first hour after administration.

Previous observations which originally led to the assumption that penicillin K is rapidly destroyed *in vivo* are worthy of critical analysis. If true, the occurrence of destruction would almost necessarily militate against the use of penicillin K therapeutically. In contrast, if penicillin K is largely bound by plasma protein *in vivo,* even though the drug is inactive when bound, it would eventually become free and potentially active as a result of the reversibility of the process of binding. It is obviously of importance to know more exactly the pharmacologic behavior of penicillin K, for a penicillin which is *not* rapidly eliminated by the kidney unquestionably would have distinct advantages, provided it were not more susceptible to destruction *in vivo*. The observations reported here and those of Richardson and his associates[9] strongly suggest that whatever mechanisms are in operation to bring about the fall in serum concentration of penicillin K after intramuscular injection, they do not remove penicillin K more rapidly than does the process of urinary excretion in the case of the other penicillins.

One might anticipate from these findings that the actual mode of administration of penicillin K could profoundly affect its comparative therapeutic effectiveness. It is of interest in this respect to contrast the results obtained by Hobby and her associates[3] and by Eagle[4] in the treatment of experimental hemolytic streptococcal infections in mice. The same infecting organism was used by both investigators. Eagle treated the animals with 10 equal doses of penicillin in saline solution administered at intervals of 3 hours. In contrast Hobby employed only 3 injections of penicillin suspended in peanut oil, and gave the injections as follows: (1) 40% of total dose immediately after infection, (2) 40% of total dose 6 hours later, and (3) 20% of the total dsoe 16 hours after the second injection. When penicillin X and penicillin G were compared in this way both investigators found virtually the same unitage ratios of X to G, *e.g.,* 480:100 (Hobby) and 500:100 (Eagle). Comparison of G with K, however, showed a marked difference between the 2 methods of study. On a unitage basis, Eagle found the G:K ratio to be 100:7, whereas Hobby found it to be 100:60. Several factors may contribute to the difference in relative effectiveness of penicillin K observed by the 2 investigators. It would appear, however, that the chief differences between the 2 studies were the size of the individual doses, and the interval between injections. Penicillin K appeared to be significantly more effective as compared with penicillin G when given in larger individual doses at longer intervals. This is in accord with the facts that penicillin K is bound to a high degree by albumin and has a low renal clearance.

The data presented on penicillin dihydro F are the first studies reported in humans. Penicillin dihydro F in general exhibited a higher degree of binding than penicillin G, and its lowest measurable level in serum was intermediate between penicillins G and K. The calculated rate of fall in serum concentration of penicillin dihydro F is slightly greater than that of G, but the difference is probably not of significance. In this respect it should be mentioned that the crystalline sample of penicillin dihydro F used in these studies may have contained 10-20% of "K-type" penicillins. Preliminary experiments on certain mixtures in serum of 2 penicillins which are bound to greatly different degrees suggest that the observed activity is solely that of the penicillin which is bound to the lesser degree. For example, pooled serum containing mixtures of penicillins G and K over a wide range of ratios of concentration, when assayed by the technic employed in these studies has consistently shown an apparent activity equal to that of the penicillin G present. Thus the values obtained for serum concentration after administration of penicillin dihydro F may actually represent what would be obtained after approximately 80% of the dose were given in the form of pure material. This probably would not differ significantly from the results which have been presented.

Summary. The absorption and excretion of penicillins X, G, dihydro F and K in humans have been studied in an effort to evaluate the influence of the marked differences in the degrees of binding of these substances by serum protein.

The duration of the serum concentrations afforded by all 4 penicillins after intramuscular injection of 300,000 units to humans were uniform within the limits of the method of assay. During the period when all of the penicillins were present in measurable concentrations there was no difference among the 4 except for the lower peak of penicillin K 15 minutes after injection. Evidence was presented that previous findings of higher and more prolonged serum concentrations of penicillin X, and lower, rapidly disappearing concentrations of penicillin K were artifacts created by differences in the antagonistic action of the serum on penicillin during the actual bioassay procedure. Thus differences in therapeutic effectiveness of the individual penicillins cannot be explained on the basis of differences in height and duration of serum concentrations after equal doses.

Although the total excretion of penicillin K was low, the rate of excretion and the renal clearance suggest that the mechanism of removal of penicillin K by the kidney differs from that observed with the other penicillins.

15899

Antidotal Properties of Crude *Penicillium notatum* Filtrate.

J. SMOLENS, D. S. MCALEER, AND C. S. MCLAREN. (Introduced by M. Landy.)
From the Wyeth Institute of Applied Biochemistry, Philadelphia, Pa.

Recent work of Ramon, *et al.* shows that there is present in filtrates of crude *Penicillium notatum* an agent which detoxifies various toxins.[1-3] This property they have called "antidotal." According to these workers this action is enzymatic and they conclude that preparation of highly purified penicillin results in a loss of this factor which, from a therapeutic point of view, may be unwise. No *in vivo* therapy data were given. The following is a summary of *in vitro* and *in vivo* experiments determining the antidotal efficacy of crude penicillium broth.

Materials. Toxin—crude tetanus which assayed about 100,000 LD/ml.

P. notatum filtrate—a crude commercial filtrate from which the penicillin had been extracted.

Mice—white, inbred strain, either sex, 18-21 g.

Broth—1% peptone.

Experimental. To make the experiment

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[1] Ramon, G., Richou, R., and Ramon, P., *C. R. acad. sci.*, 1946, **222**, 621.

[2] Ramon, G., Richou, R., and Ramon, P., *La Presse Medicale*, 1946, Oct. 2.

[3] Ramon, G., Richou, R., and Ramon, P., *Rev. Immunol.*, 1944-45, **9**, 161.

TABLE I.

Test	Tube				
	1	2	3	4	5
Material					
Tetanus toxin (3 ml)	1:100,000 dil.	1:25,000	1:5,000	1:1,000	0
Original filtrate or dialyzed filtrate (ml) or dialysate	3	3	3	3	3
Broth	0	0	0	0	3
Toxin control					
Toxin (3 ml)	1:100,000	1:25,000	1:5,000	1:1,000	
Broth (ml)	3	3	3	3	

inclusive 3 materials were obtained; (a) original crude penicillium filtrate, (b) dialyzed filtrate, (c) dialysate. The dialysis was carried out at 1°C. (b) was obtained by dialyzing against running distilled water for 96 hours. The dialysate (c) was obtained by immersing in a cylinder of distilled water, a cellophane tube containing filtrate, and allowing it to stand for 48 hours. A total of 300 ml filtrate was used for (c).

The above materials were utilized in *in vitro* and *in vivo* experiments in order to determine the efficacy of the antidotal factor. If the active material is dialyzable then the agent is of relatively small molecular size.

Total solid determinations based on corrected volumes for dialysis, gave the following results: (a) original filtrate—34 mg per ml, (c) dialysate—15 mg per ml.

In vitro neutralization test, the scheme in Table I was used.

Three sets were made for each test and controls. All toxin dilutions were made with 1% peptone broth. One set each of each test and control was kept at 2°C, 20°C, and 37°C for 16 hours. Three mice per dilution were then injected intra-abdominally with 1 ml volume each. The results are shown in Table II.

Table II shows that the toxin is completely inactivated by the agent which is dialyzable. The fact that it will dialyze indicates that it is not a protein and therefore the action is not enzymatic.

There appears to be a lethal factor present in the dialyzed filtrate which is not present in the undialyzed filtrate.

Effect of blood on antidotal activity. Two ml of dialysate was added to 2 ml of sterile sheep's blood. Two ml of tetanus toxin containing 6 L.D. per ml was added and the contents mixed. Two sets were made, one being placed at 2°C and one at 37°C for 2 hours. Five mice were injected, subcutaneously to the right of the tail, with each mixture using 1 ml volume per mouse. Control mice received the same mixture without toxin. All the test mice died within 3 days with typical tetanus symptoms. All control mice survived.

The above experiment was repeated except that the incubation period at 2°C and 37°C was for 6 hours. All of the test mice died within 3 days corroborating the above results.

Acute toxicity in mice of antidotal material. The mouse toxicity of the original clear filtrate was determined since it was desirable to utilize optimal nontoxic doses of filtrate and dialysate in the *in vivo* therapeutic experiments. When the intra-abdominal route was used the LD_{50} of the original filtrate was found to be about 177 mg per 18-21 g mouse. The M.L.D. of the dialysate was >76 mg.

In vivo therapeutic test. Five mice were each injected intra-abdominally with 4 L.D. of tetanus toxin. Simultaneously, these mice received 0.5 ml of crude undialyzed filtrate subcutaneously. Two more subcutaneous injections of the filtrate at 2-hour intervals were given. Since each ml contained 34 mg a total of 51 mg was given. All 5 mice died within 2 days. Control mice which received only the filtrate survived.

Another group of 15 mice was injected subcutaneously with 2 L.D. tetanus toxin just to the right of the tail. This was followed

TABLE II.

Material	Incubation temp. °C	Toxin dilution, × 1000	Day of death	
Undialyzed*	2	1:100		
		1: 25		
		1: 5		
		1: 1		
		No toxin		
	20	1:100		
		1: 25		
		1: 5		
		1: 1		
		No toxin		
	37	1:100		
		1: 25		
		1: 5		
		1: 1		
		No toxin	2	
Dialyzed*	2	1:100	333	46
		1: 25	222	223
		1: 5	111	111
		1: 1	111	111
		No toxin	222	
	20	1:100	334	44
		1: 25	222	222
		1: 5	111	111
		1: 1	211	111
		No toxin	223	
	37	1:100	334	12
		1: 25	222	145
		1: 5	111	222
		1: 1	211	111
		No toxin	223	
Dialysate	2	1:100		
		1: 25		
		1: 5		
		1: 1		345
		No toxin		
	20	1:100		
		1: 25		
		1: 5		
		1: 1		
		No toxin		
	37	1:100		
		1: 25		
		1: 5		
		1: 1		
		No toxin		
Toxin controls*	2	1:100	36	443
		1: 25	222	222
		1: 5	211	111
		1: 1	111	111
	20	1:100	344	444
		1: 25	222	24
		1: 5	111	111
		1: 1	111	111
	37	1:100	446	446
		1: 25	222	222
		1: 5	222	111
		1: 1	—	111

* Results for undialyzed, dialyzed, and toxin controls are based on 2 tests or total of 6 mice per dilution.

immediately with 1 ml dialysate intra-abdominally. One ml of dialysate was given intra-abdominally every hour for 13 hours with a 13-hour interval between the 6th and 7th injections. A total of about 220 mg per mouse was given. All of the mice developed typical tetanus symptoms in about 24 hours and all of the mice died in 2 or 3 days. Five control mice which received only the dialysate showed no ill effects.

Test for destruction of antigenicity of toxin. Since the *in vivo* experiments showed the *P. notatum* broth to be without therapeutic activity it was not known whether there was *in vivo* destruction of the agent. However, the "antidotal" factor might prove to be a valuable material for preparation of toxoids. To test this possibility the 36 mice which had received undialyzed filtrate mixed with toxin and survived were challenged by intra-abdominal injection of 4 L.D. of tetanus toxin 21 days following the original test. All 36 mice died within 2 days. These results were corroborated in a similar experiment in which 66 surviving mice were challenged after 21 days with 4 L.D. of tetanus toxin. All the mice died within 3 days. Thus it would seem that the agent in the filtrate not only detoxifies toxin but also destroys antigenicity. This evidence is admittedly indirect but since the tetanus toxin used in these experiments represents an aliquot from a large batch of toxin used for making satisfactory commercial toxoid it is felt that the results are valid.

Determination of antidotal activity in amorphous penicillin. Results similar to those obtained with crude filtrate against tetanus toxin were observed with amorphous penicillin calcium having a potency of 1000 units per mg. *In vitro* neutralization occurred but no *in vivo* therapeutic activity was demonstrated.

Antidotal activity versus the endotoxin of H. pertussis. Four concentrations of *P. notatum* filtrate in a volume of 3 ml were made; undiluted, 1:4, 1:16 and 1:64. The diluent was peptone broth. Three ml of pertussis endotoxin* containing 50 LD_{50} per ml were added to each concentration of filtrate. The contents of the tubes were mixed and kept at 2°C for 6 hours. For each group 5 mice were then injected intra-abdominally with 1 ml volume. There were no deaths in the groups where undiluted or 1:4 filtrate was used. In the 1:16 and 1:64 dilutions all of the mice died.

In vivo experiments identical to those described previously for tetanus toxin (intra-abdominal route) were carried out using 4 L.D. of pertussis endotoxin. All of the mice died.

The *in vitro* and *in vivo* results obtained with pertussis endotoxin were the same as those observed with tetanus toxin. It would appear that the *in vitro* antidotal activity is not specific.

Discussion. Ramon, Richou, and Ramon did not include any *in vivo* results in their communications. However, the *in vitro* results reported seemed to warrant the foregoing experiments. Both the *in vitro* and *in vivo* data contained in this report are very striking in the definite results attained. The fact that blood neutralizes the antidotal activity is in itself, sufficient evidence to predicate failure of *in vivo* therapy.

With the *in vitro* incubation period of 16 hours used in the present experiments there seems to be no substantial difference whether the incubation temperature is 2°C, 20°C, or 37°C.

Summary. 1. The *in vitro* results on the antidotal factor reported by Ramon, *et al.* are essentially corroborated.

2. This antidotal agent is dialyzable.

3. The antidotal activity is inactivated in the presence of blood.

4. No *in vivo* therapeutic activity against tetanus toxin or pertussis endotoxin could be demonstrated.

Both the crude penicillium filtrate and the amorphous penicillin calcium were kindly supplied by the Penicillin Division of Wyeth, Inc. We are indebted to the Biological Division of Wyeth, Inc. for the crude tetanus toxin.

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* For the method of preparation see Smolens, J., and Flavell, E. H., *J. Immunol.*, in press.

15900

Influence of Arginine on Oligospermia.

Henry S. Guterman. (Introduced by R. Levine.)

From the Department of Metabolism and Endocrinology, Michael Reese Hospital Research Institute, Chicago, Ill.*

Shettles observed that oligospermia, occurring in men fed an arginine-deficient diet, was relieved when arginine was returned to the diet.[1] Since the therapeutic approaches to oligospermia have been unsatisfactory, it appeared that arginine might prove helpful in clinical cases of oligospermia.

The present report is an account of observations made in this laboratory on 23 patients with oligospermia. Eighteen received large amounts of pure arginine and minimal quantities of lysine, pyridoxine, and tryptophane as supplements to their usual diet. Five patients received no specific therapy.

Methods. A complete medical history and physical examination in each of the 23 patients studied failed to reveal any known etiological factors contributing to the oligospermia. Thirteen had received previous hormonal therapy which failed to influence their low sperm counts. Ten men whose oligospermia was discovered during the present study had received no previous therapy.

The patients' semen was examined every 4 weeks for volume, sperm-motility, and concentration of spermatozoa according to the method of Macomber and Saunders.[2] The specimens were obtained by masturbation or coitus interruptus after the patients had abstained from intercourse for 4-5 days.

After the semen specimens were studied from 1-3 months, the patients ingested daily, as uncoated tablets, 1.8-2.7 g arginine and 40-60 mg each of lysine, pyridoxine and tryptophane in addition to their usual diet.† Therapy lasted for 8 months and terminated after that interval in most cases because the sperm concentration had not risen significantly.

Table I summarizes the results of the study and includes pertinent clinical data as well as the sperm counts observed.

Group I includes 5 patients who were studied before, during, and after amino acid therapy. The initial sperm count of these men averaged 12.9 million per cc. One month later, just prior to the amino acid therapy, the average concentration was 11.2 million per cc. After 8 months of treatment, the average sperm count had increased to 47.0 million per cc. The amino acids were then discontinued and 8 months later, the sperm counts averaged 28.1 million per cc. Although these patients received much larger quantities of arginine than Shettles' subjects, their sperm counts did not increase rapidly or to normal levels as Shettles observed in his arginine-deprived men.[1] One patient in our group developed a normal count (100 million per cc) during treatment, but conception did not occur.

The 7 patients in Group II demonstrated a drop in sperm count (from 26.0 to 12.4 million per cc) in the 3-month pretreatment period. After taking arginine for 7 months, these men had an average sperm count of 21.8 million per cc. Since the sperm concentration under arginine did not even attain the initially low levels, it appears that the response noted was not significant. None of these patients' wives became pregnant during the study.

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* The department is in part supported by the Michael Reese Research Foundation.

[1] Shettle, L. B., *Proc. Third Annual Conference on Biology of the Spermatozoa*, pp. 28-36, 1942, National Committee on Maternal Health, Inc., New York.

[2] Macomber, D., and Saunders, W. B., *New England J. Med.*, 1929, **200**, 981.

† The amino acids were generously supplied by Dr. Clair Folsome, Ortho Research Foundation, Raritan, N.J.

TABLE I.
Summary of Results.

Group	No. of pts.	Age	Infertility (years)	Arginine treatment (mo.)	Conceptions	Sperm counts (× 10⁶ per cc)			
						Initial	Pretreatment	At end of treatment	Final after treatment discontinued
I	5	35	5.4	8	0	12.9 (.4-22)	11.2 (0-18)	47 (15-100)	28.1 (.7-45)
II	7	34	4.0	7	0	26.0 (0-75)	12.4 (0-30)	21.8 (0-70)	—
III	6	32	4.1	8.8	0	—	17.3 (5-46)	26.4 (1.5-57)	—
IV	5	36	5.5	—	2	20.3 (0 35.5)	40.2 (0-104)	—	—

The numbers indicate the averages for the group. The numbers in parentheses indicate the ranges observed.

The oligospermia of the 6 patients in Group III had been present 1.5-9 years. They received arginine after the first semenalysis in this laboratory, and 8 months later the average sperm count had risen to 26.4 million per cc from the initially recorded 17.3 million per cc. No pregnancies occurred in this group during the study.

The sperm counts of the 5 men in Group IV rose during the period prior to contemplated therapy. These patients did not receive the amino acids, since the spontaneous increase in sperm concentration might have been interpreted as a direct result of treatment. In the average 3-month period of study, the sperm counts of these men rose from an average of 20.3 to 40.2 million per cc. The wives of two of the patients with low counts became pregnant in this period. Although one patient attained a sperm count of 140 million per cc, conception did not occur.

Discussion. Arginine (with small amounts of lysine, pyridoxine, and tryptophane) did not solve the primary problem of the 18 treated infertile patients in this study. The increase in sperm counts of the untreated control group was as great as that of the treated group. None of the wives of the treated subjects became pregnant during the study, while 2 pregnancies occurred among the couples of the untreated group.

All the patients in this series gave a history of adequate protein intake. Shettles' studies on the beneficial effects of arginine on oligospermia were carried out on men and animals who became oligospermic on an arginine-deficient diet and then returned to normal status in a few weeks with the addition of arginine to the deficient diet.[1] In view of the results obtained in the present study, it appears that the beneficial action of arginine on impaired spermatogenesis is specific for oligospermia due to arginine deficiency. Its use for this purpose should be reserved for patients with oligospermia who exhibit or give the history of a deficient intake of arginine.

Summary. 1. Eighteen infertile men with oligospermia received 1.8-2.7 g of arginine and minimal amounts of lysine, pyridoxine, and tryptophane in tablet form for 8 months.

2. The tendency for sperm concentration to increase was as great in the untreated group as in the treated groups.

3. Although no pregnancies occurred in the treated groups, 2 pregnancies were recorded among the wives of 2 of the control subjects.

4. It appears that amino acid therapy for oligospermia should be reserved for those patients who exhibit or give the history of inadequate protein intake.

The author wishes to acknowledge with thanks, the aid and encouragement of Dr. R. Levine, Director of the Department of Metabolism and Endocrinology, in this work. Dr. A. Koff was most cooperative in referring patients for study.

15901 P

Effect of Para-Aminobenzoic Acid on Fever and Joint Pains of Acute Rheumatic Fever.

Herman Rosenblum and L. E. Fraser. (Introduced by Douglas H. Sprunt.)

From the Division of Pediatrics of the College of Medicine, University of Tennessee, Memphis, Tenn.

Para-aminobenzoic acid has been shown to be beneficial in the treatment of the rickettsial group of diseases.[1-4] With the idea in mind that acute rheumatic fever shows a vasculitis[5] which is similar in some respects to that produced by the rickettsial diseases, and that both diseases are again similar in that they may be aggravated by the use of the sulfonamides,[6-8] para-aminobenzoic acid has been used experimentally to determine what effect, if any, it would produce on the fever and joint pains of acute rheumatic fever. There is one report[9] in which it was used to sustain the salicylate level in the blood, and in which case the para-aminobenzoic acid, when used alone, apparently exerted no effect for the time during which it was administered.

Material and Procedure. Nine patients in which the diagnosis conformed to accepted criteria[10] have been given the drug. These were all colored children ranging in age from 6 to 12 years. Eight of them had joint pains. In some cases they were mild and in others they were severe to the point of incapacitation. Sickle cell anemia was carefully excluded in each instance.

Para-aminobenzoic acid powder was used and an immediate dose of 3-4 g was given, followed by a maintenance dose of 1-3 g given at 2-3-hour intervals. Blood levels were taken within an hour of the administration of the drug. Alkalis were given to prevent the development of acidosis. Salicylates, penicillin, and sulfonamides were withheld, except prior to the establishment of a definite diagnosis, or when they were necessary

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[1] Yeomans, A., Murray, E. S., Zarafonetis, C. J. D., and Ecke, R. S., *J. Am. Med. Assn.*, 1944, **126**, 349.

[2] Tierney, N. A., *J. Am. Med. Assn.*, 1946, **131**, 280.

[3] Smith, P. K., *J. Am. Med. Assn.*, 1946, **131**, 1114.

[4] Flinn, L. B., Howard, J. H., Todd, C. W., and Scott, E. G., *J. Am. Med. Assn.*, 1946, **132**, 911.

[5] Von Glahn, W. C., and Pappenheimer, A. M., *Am. J. Path.*, 1926, **2**, 235.

[6] Topping, N. H., *Pub. Health Rep.*, 1939, **54**, 1143.

[7] Swift, H. F., Moen, J. K., and Hirst, G. K., *J. Am. Med. Assn.*, 1938, **110**, 426.

[8] Massell, B. F., and Jones, T. D., *New Eng. J. Med.*, 1938, **218**, 876.

[9] Dry, T. J., Butt, H. R., and Scheifley, C. H., *Proc. Staff Meet., Mayo Clin.*, 1946, **21**, 497.

[10] Jones, T. D., *J. Am. Med. Assn.*, 1944, **126**, 481.

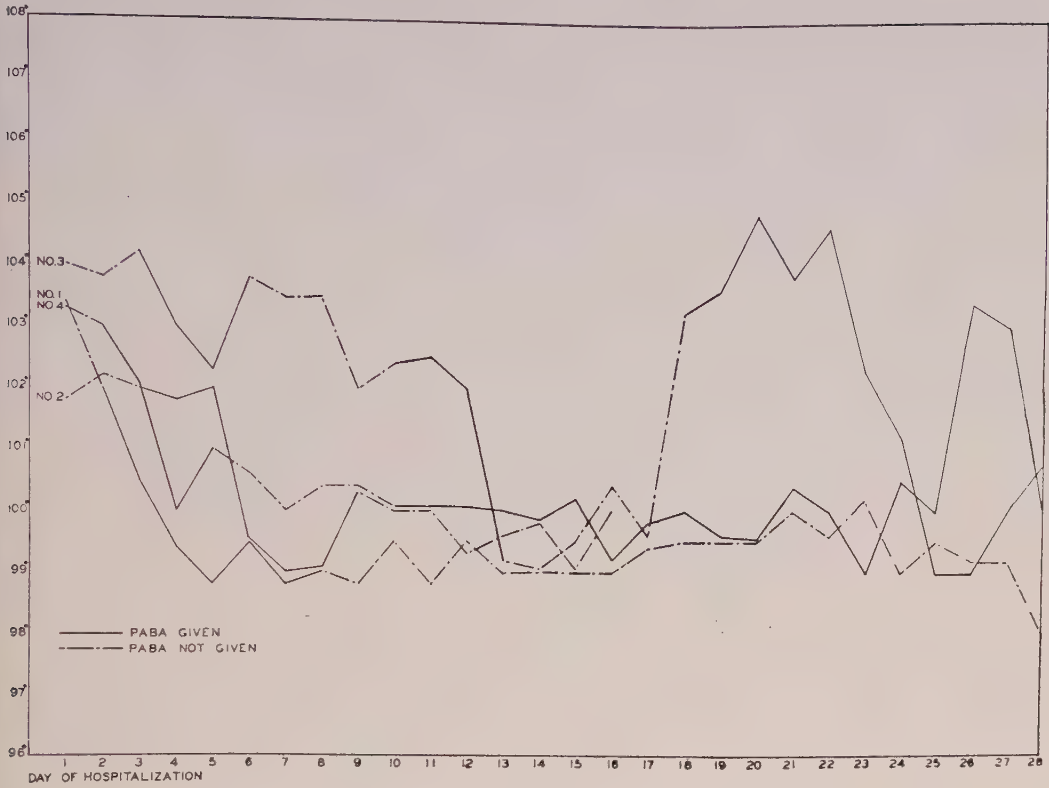


Chart 1.

to treat intercurrent complications. All temperatures were taken rectally.

Results. In 7 of the cases, which received para-aminobenzoic acid on the 1st or 2nd hospital day, there was apparently a prompt and definite response characterized by a fall in temperature and relief of the joint pains. The temperature became normal in these cases on the 3rd to the 7th day (Charts 1 and 2) and the joint pains were relieved on the 2nd to 5th days after the drug was started, usually on the 2nd or 3rd day. In Case No. 3, sulfadiazine was given for the 1st hospital day only, penicillin was given from the 2nd through the 7th day, and salicylates were given from the 7th through the 14th day. No response was obtained under this therapy. During this time para-aminobenzoic acid was given for 3 days, but in probably inadequate dosage. Following the reinstitution of this drug on the 11th day the temperature dropped to normal on the 13th day, when a level of 173.2 mg per 100 cc was obtained. It is possible that the effect in this case may have been due to a combined action of salicylates and para-aminobenzoic acid. In Case No. 7, there seemed to be a sustained suppression of the temperature while the drug was being given, followed by a prompt rise when it was discontinued and an immediate drop when it was reinstituted. In this case the joint pains persisted but were vague. In Cases No. 5, 6, 7, and 8 the only drug used was para-aminobenzoic acid. Case No. 9 received penicillin on the 2nd hospital day only, in addition to the para-aminobenzoic acid.

Three patients, Cases No. 3, 4, and 6 had secondary rises of temperature while taking the drug. Case No. 3 had an elevation for 6 days continuously, No. 4 for 5 days and No. 6 for 11 days. All of these patients retained a sense of well being out of proportion to their temperature. The throat was found to be moderately inflamed in Case No. 4 which may have accounted for his eleva-

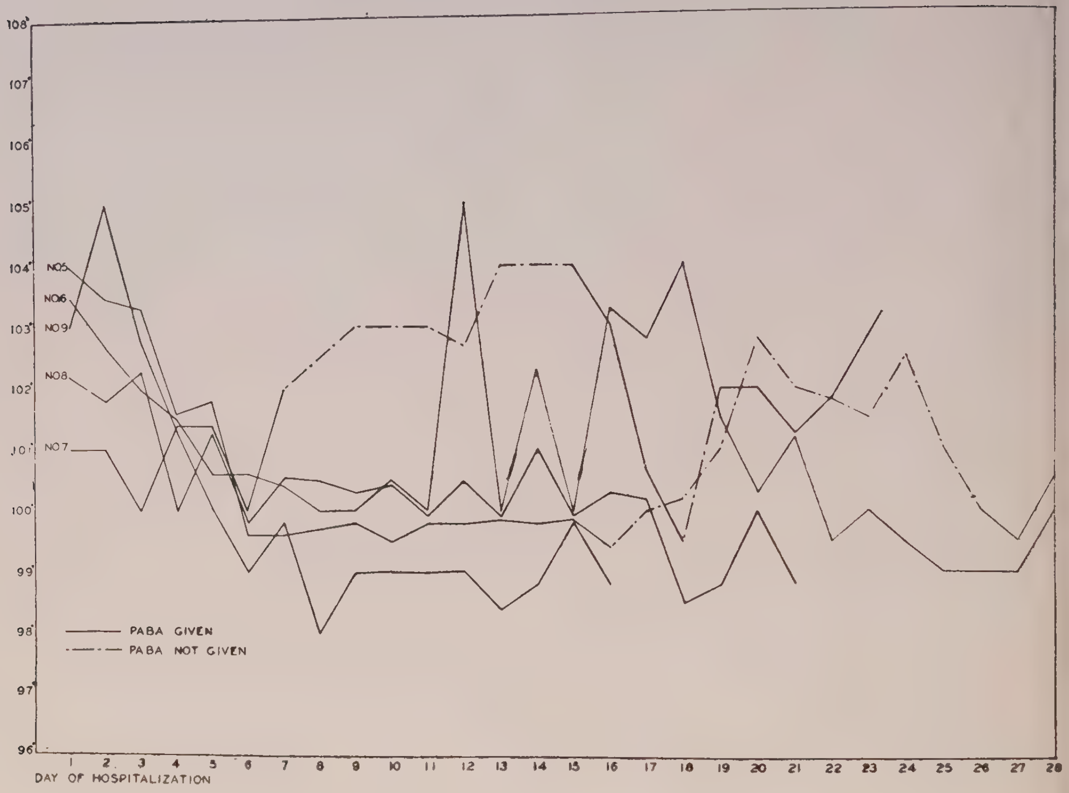


Chart 2.

tion. An unidentified reducing substance was found in the urine in the majority of these cases.

Conclusions. Para-aminobenzoic acid, in the small series of 9 cases of acute rheumatic fever presented, appeared to have a definite effect on the fever and joint pains.

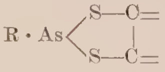
15902

Effect of BAL on Trichomonacidal Activity of Some Organic Arsenicals.

Ernst A. H. Friedheim and Rose L. Berman. (Introduced by M. B. Sulzberger.)

From the Laboratory of E. A. H. Friedheim, New York City.

The inclusion of an aromatic trivalent arsenic radical in a 5-membered ring of the type

I 

$$R \cdot As \left\langle \begin{matrix} S-C= \\ \quad | \\ S-C= \end{matrix} \right.$$

does not preclude a chemotherapeutic effect with an index of the same order of magnitude, characteristic for the parent compound

II R · AsO

This conclusion was reached in a previous study on the trypanocidal effect of the condensation product of Mapharsen and BAL, *i.e.*, 2-amino-4-[methylolcyclo-(ethylenedimercaptoarsino)]-phenol hydrochloride[1]

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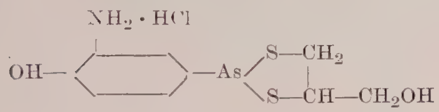
[1] Friedheim, E. A. H., and Vogel, H. J., Proc. Soc. Exp. Biol. and Med., 1947, **64**, 418.

TABLE I.
In Vitro Effect of Some Arsenic and Antimony Compounds and Bromoacetic Acid on *Trichomonas vaginalis.*
Results of Microscopic Dark Field examinations of *Trichomonas vaginalis* from Clinical Cases, Suspended in Drug Solutions.

Drug	Final drug dilution				
	1:100	1:200	1:400	1:600	1:800
Mapharsen BAL[1]	X	X	X	X	0
Mapharsen	0	0	0	0	0
70 A BAL	X	0	0	0	0
70 A	X	0	0	0	0
p-Melaminylphenyl sodium arsonate[2]	0	0	0	0	0
p-Melaminylphenyl sodium stibonate[3]	X	0	0	0	0
Aldarsone	0	0	0	0	0
Acetarsone (Stovarsol)	0	0	0	0	0
Bromoacetic acid	0	0	0	0	0

X = Complete immobilization of trichomonas in less than 5 min.
0 = No or incomplete immobilization in less than 5 min.

III 

in the *T. equiperdum* infection of the mouse. The same principle has now been found to apply equally well and more so to the immediate toxic *in vitro* effect of III on *Trichomonas vaginalis.*

Observations summarized in Table I show that III not only equals but surpasses significantly the damaging effect of Mapharsen on *Trichomonas vaginalis,* as measured by the drug concentration required to immobilize the parasites within a chosen time interval. Taking 5 minutes as the critical observation time, Mapharsen is inactive in a dilution of 1:100, while its BAL derivative III is active at 1:600.

Considering the possible mechanism of this effect, we note that the condensation with BAL eliminates from the parent molecule a hydrophilic group, *i.e.,* the polar arsenoso radical, and introduces a lipid-soluble group, *i.e.,* the BAL radical. Both changes concur in affecting the water/lipid distribution coefficient in favor of the lipid phase. Thus condensation of Mapharsen with BAL allows for a new distribution of the $R \cdot As$– radical, permitting the arsenic to come in contact with biochemical systems it could not reach before. The hypothesis may be formed that this new distribution based on relative lipid solubility is responsible for the enhanced toxic effect of the BAL derivative on this particular parasite.

This conception is sustained by the observation on compound 70A, Eagle, (γ-(-arsenosophenyl)-butyric acid), and the corresponding BAL derivative, reported in Table I, although here the condensation with BAL fails to exert a significant effect on the trichomonacidal activity. This result seems at first glance to contradict the hypothesis under discussion, but at closer scrutiny, is well in keeping with it: The butyric acid residue, characteristic for this compound, impresses on the molecule a strongly polar, anionic character, which dominates even after condensation with BAL, precluding the degree of lipid solubility necessary for a trichomonacidal effect.

Table I includes a comparative control of the BAL experiments with 3 standard as well as with 2 new organometallic compounds. It follows that under the chosen experimental conditions, the clinically much used pentavalent arsenic compounds Acetarsone, Aldarsone,* and the metal-free bromoacetic acid are inactive. Equally inactive is a new arsenical, *p*-melaminylphenyl sodium arsonate,[2] but it is very noteworthy that the analogous antimony compound, *i.e.*, *p*-melaminylphenyl sodium stibonate[3] is of significant activity.

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* Acetarsone and Aldarsone failed to immobilize trichomonas even at the highest concentration chosen, *i.e.,* 1:100, within 30 minutes. The mobility of the trichomonas in the suspension continued unabated for 6 hours.

Experimental. Material. Fresh specimens of vaginal secretion from clinical cases of heavy *Trichomonas vaginalis* infestation were suspended in saline. The trichomonas suspension containing 4-12 parasites per field (obj. 20X; ou. 18X) was mixed in test tubes with equal parts of drug solution adjusted to pH 6-7.8. The mixing, the preparation of the slide, and the first microscopic observation consumed on the average 25 seconds. Samples of the mixture were examined after various intervals as to the presence of *mobile* parasites in at least 100 fields.

Criteria for Activity. "Immobility" is here defined as "absence of movement of flagella and undulating membrane" in all parasites encountered. In most preparations, the cessation of movement was followed rapidly by a disintegration of the parasites. In other cases, the immobilization could not with certainty be considered as the expression of a lethal effect. The term "trichomonacidal" is used in the present paper with this restriction.

Compound 70A. γ-(*p*-arsenosophenyl)-butyric acid, was kindly supplied by Dr. Eagle. The BAL derivative was prepared by reacting equal molar quantities of the 70A sodium salt and BAL in an aqueous medium.

The reaction product, *i.e.*, γ-4-[methylolcylco-(ethylenedimercaptoarsino)]-phenylbutyric acid, crystallizes in fine white needles. It is soluble in alkali, ethanol, acetone and propylene glycol, insoluble in water and dilute mineral acids. The nitroprusside test for SH groups is negative at pH 8, but positive in the presence of an excess of free alkali.

Analysis.

Calcd. for $C_{13}H_{17}O_3S_2As$: As, 20.8%
Found : As, 20.5%

Summary. 1. The condensation with BAL enhances significantly the trichomonacidal effect of Mapharsen.

2. The resulting compound, *i.e.*, 2-amino-4-[methylolcyclo-(ethylenedimercaptoarsino)]-phenol, in the form of its hydrochloride, compares favorably with acetarsone, aldarsone, and bromoacetic acid as to trichomonacidal effect.

3. The increase of trichomonacidal activity brought about by condensation with BAL is considered as associated with the concurrent increase in lipophilic character.

4. A comparison between the arsonic and the stibonic acid derived from the identical organic radical shows the arsenic compound to be inactive and the analogous antimony compound to be active and superior to acetarsone and aldarsone as to trichomonacidal effect.

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[2] Friedheim, E. A. H., *J. Am. Chem. Soc.*, 1944, **66**, 1775.

[3] Friedheim, E. A. H., Vogel, H. J., and Berman, R. L., *J. Am. Chem. Soc.*, 1947, **69**, 560.

15903

Clinically Apparent and Inapparent Infection with Japanese B Encephalitis Virus in Shanghai and Tientsin.

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The occurrence of the Japanese B type of encephalitis in Peiping, China, has been reported in recent years at first on the basis of generally inconclusive neutralization tests,[1–4] and later in 1941 on the basis of unequivocal identification of a virus recovered from a fatal case.[5] During the summer of 1946, an outbreak of an illness among U. S. Marines stationed in Tientsin which was at first thought to be encephalitis and later regarded as poliomyelitis, led to certain investigations which are reported in this communication. Since we could obtain no history of outbreaks of "summer" encephalitis in Tientsin, it was deemed desirable to determine by means of neutralization tests whether or not there was evidence of inapparent dissemination of the virus in the native population. Accordingly, blood was obtained from 20 Chinese adults, aged 17 to 48, who had no history of encephalitis and stated that they had spent their entire lives in the Tientsin area. During this period there were also submitted to our laboratory acute and convalescent serum specimens from a case of acute encephalitis in a Chinese doctor in Shanghai, and, through the courtesy of Dr. Ming-Sing Hwang, we subsequently obtained blood from a group of Chinese adults in Shanghai who had no history of encephalitis.

Clinically Apparent Infection in Shanghai. According to Dr. M. S. Hwang, there is no record of an outbreak of encephalitis in Shanghai, although infrequent sporadic cases are seen,[6] and to the best of his or our knowledge, no case of encephalitis in Shanghai had been proved as being caused by the Japanese B encephalitis virus. During the summer of 1946, Dr. Hwang saw 2 other cases of acute encephalitis in addition to the patient to be reported here. While we were in Shanghai in the middle of August, 1946, Dr. Hwang "combed" the crowded hospitals for cases of acute encephalitis but all we were able to find on rounds were a few cases, in which the most probable clinical diagnoses were poliomyelitis, tuberculous meningitis, and measles encephalopathy. We are indebted to Major F. A. Mantz, M.C. of the Surgeon's Office of the China Service Command

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‡ Attached to the Commission while on active duty in the Army.

§ We wish to express our indebtedness to Brig. Gen. S. Bayne-Jones, at the time deputy chief of the Preventive Medicine Service, Office of the Surgeon General, U. S. Army, Brig. Gen. J. I. Martin, U.S.A., Chief Surgeon of the U. S. Army Forces in the Pacific, Col. Crawford F. Sams, M.C., chief of the Public Health and Welfare Section of the Supreme Command for the Allied Powers, and Lt. Col. W. D. Tigertt, M.C., commanding officer of the 406th Medical General Laboratory, for making possible the work of this Commission.

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TABLE I.
Complement Fixation Tests on Acute and Convalescent Sera from a Case of Encephalitis (Dr. O. H. J.) in Shanghai (July, 1946).

				Complement fixation in mixtures with indicated antigens																
			Serum days after onset	Japanese B Serum 1:*							St. Louis Serum 1:				WEE Serum 1:		Saline Serum 1:		Jap B C-F Titer
Test	Exact units of complement	Temp. inactiv.		2	4	8	16	32	64	128	2	4	8	16	2	4	2	4	
I	1.7	60°C	7	4	4	4	4				3	1			1	0	2		AC
			21	4	4	4	4				4	2			±	±	±		AC?
II	1.7-2.0	65°C	7		4	2	1	±	0	0		0	0	0		0		0	1:8
			21		4	4	4	2	1	1		±	0	0		0		0	1:32

WEE = Western equine encephalitis virus. AC = Serum anticomplementary.

Saline = Mixture with physiological salt solution instead of antigen to check on anticomplementary properties of the serum.

* The serum dilutions indicated here are the original dilutions added to the mixture and should be multiplied by 4 for the final dilution in the mixture prior to the addition of the sensitized cells. In test II the serum dilutions began at 1:4 because there was not enough serum to start with lower dilutions.

4 = Complete fixation; 3,2,1 = Different degrees of partial fixation; ± = Questionable trace; 0 = No fixation, or complete hemolysis. The original dilution of serum giving 2 plus (approximately 50%) fixation represents the titer.

and Dr. M. S. Hwang for the clinical history and serum specimens of the patient to be reported.

The patient was Dr. O.H.J., a 29-year-old male, Chinese physician who had not been outside of the environs of Shanghai in "recent" months; he was attending physician at a tuberculosis clinic and in "robust" health until the onset of the present illness. On 12 of July, 1946, 24 hours after the extraction of several teeth, there was a sudden onset of severe, generalized headache and fever. The headache became progressively worse and upon admission to the hospital on 14 of July he was said to be distinctly somnolent, and except for the fever (102°-104°F by mouth) and a relative bradycardia (90-110), the general physical and neurological examinations and an X-ray film of the chest revealed no abnormalities. Daily examinations of the cerebrospinal fluid showed a pleocytosis ranging between 195 and 300 cells per cu mm with 79 to 87% lymphocytes, sugar of 78 to 95 mg %, negative Pandy and Nonne-Appelt tests for protein, and no bacteria on smear. On 17 of July, instead of somnolence the patient exhibited a muttering delirium with only occasional lucid moments, pronounced nuchal rigidity, left central (supranuclear) facial palsy, somewhat hyperactive reflexes, and the Babinski sign was positive bilaterally. A well marked ophthalmoplegia was subsequently noted on the left side. On 19 of July the fever, relative bradycardia and general condition were still the same, and although the diagnosis of acute encephalitis was favored, basilar meningitis was also considered and large doses of penicillin were administered between 17 and 30 of July. Improvement began about 22 of July and by 5 of August, all abnormal neurological signs had disappeared and with the exception of extreme generalized asthenia the patient appeared well and had no complaints.

The first blood specimen was obtained on 19 of July, 7 days after onset, and the serum reached the Tokyo laboratory on 24 of July when it was frozen and stored in an insulated box containing solid CO_2. The second specimen was taken 2 of August, 21 days after onset, and reached the Tokyo laboratory on 6 of August, when it was frozen in the same manner. The protocols of the complement fixation tests with these sera are shown in Table I. Both sera were somewhat anticomplementary in the first test after the routine inactivation at 60°C for 20 minutes, probably due to the lack of refrigeration during the 4- to 5-day interval prior to their arrival in Tokyo. In the second test after heating at 65°C for 20 minutes, specific fixation was demonstrable with the Japanese B virus antigen and the titer in the convalescent serum (1:32 of the original serum) was 4 times greater than that obtained with the acute specimen. The intracerebral neutralization test in mice yielded an equivocal index of 32 with the acute specimen of serum and a distinctly positive index of 80 with the convalescent serum. Thus, the results of both types of tests, but especially those of the complement fixation test, warrant a serological diagnosis of Japanese B encephalitis.

Clinically Inapparent Infection in Tientsin and Shanghai. The blood specimens from the Chinese adults without history of encephalitis were iced immediately after they were obtained, and the sera were frozen and stored in a box containing solid CO_2 4 to 6 days later. The neutralization tests were carried out with the undiluted, unheated sera against varying dilutions of the Nakayama strain of Japanese B encephalitis virus, and the results are shown in Table II. Thus, 17 (89%) of the 19 sera from Chinese residents of Tientsin, aged 17 to 57, and 11 (85%) of the 13 sera from Chinese residents of Shanghai, aged 16 to 51, contained distinctly significant titers of neutralizing antibody for the virus. In 1943, Mitamura[7] reported that 83% of 104 "normal" human sera (age not indicated but Dr. M. Kitaoka informed us that they were "mostly" adults) obtained from Middle China (Nanking and Shanghai) in 1941 neutralized the Japanese B virus, as compared with 83% of 116 human sera from Tokyo in 1936 (one year after the largest epidemic) and 0.8% of 525 human sera from Hokkaido, Japan in 1937 where no epidemic of the disease had been observed up to that time. In 1945, 90% of natives on Okinawa, aged 20 to 60, were found to have neutralizing antibodies for this virus.[8]

Our own data taken together with those reported by Mitamura indicate that the virus of Japanese B encephalitis had undergone as extensive dissemination among the population of North and Middle China, where only very few cases of encephalitis have been observed, as in those areas of Japan where the severest epidemics have occurred. Since antibodies can be detected in 80 to 90% of adults and since even the severest epidemics have attacked only a few thousand out of many millions of people, it is obvious that infection with this virus must be inapparent in the vast majority of human beings in Japan as well as in China, and the question really is whether epidemics might have occurred in China but had gone unrecognized. It is generally admitted in China, that only a small proportion of the population seeks the advice of, or is seen by, western-trained Chinese doctors and the possibility is granted that large numbers of cases of encephalitis could be missed. However, there are many good hospitals with thousands of patients in the large population centers of Shanghai, Peiping, Tientsin, etc. and an unusual increase in the number of cases during the summer and autumn, although it would only be relative rather than absolute, as far as numbers are concerned, could hardly go unrecognized. During the summer of 1938, Chu, Wu and Teng[2] saw 10 cases of acute encephalitis on the Pediatric Service of the Peiping Union Medical College within the short period of 24 days from July 29 to August 22, and 3 additional cases were seen in older individuals.[3] In 1939, 16 cases of acute encephalitis were admitted to the Peiping Union Medical College, and 12 of these came in during July, August and September.[3] During our visit to the Central Hospital in Peiping in August, 1946 we had an opportunity to see 2 patients in the acute stage of encephalitis with clinical manifestations and laboratory findings that could be entirely compatible with the Japanese B type of encephalitis. It is a pleasure to record here the excellence and completeness of the clinical and laboratory observations which we found on the patients at the Central Hospital. Dr. William H. L. Chung, the medical director and chief of the medical service at the Central Hospital, very kindly supplied us with the data on the cases of encephalitis admitted to this hospital during the 4 years of 1942 to 1945, which are shown in Table III. While the number of cases admitted during the 4 months of June to September is regularly as great or greater than during the other 8 months of the year, suggesting

TABLE II.

Intracerebral Neutralization Tests in Mice with Japanese B Encephalitis Virus on Sera from Normal Chinese Residing in Tientsin or Shanghai.

		Serum		Mortality at indicated final dilutions of virus					
City	Test	No.	Age of donor, years	10^{-6}	10^{-7}	10^{-8}	10^{-9}	LD_{50}	Neutralization index
Tientsin	A	Rabbit control		4/4	5/5	3/4	0/4	8.3	—
		1	48	1/4	0/5	0/5		5.7-?	400+
		2	35	0/5	0/5	0/5		5.5-?	630+
		3	40	3/3	4/5	1/5		7.5	6
		4	20	0/5	0/5	0/4		5.5-?	630+
		5	37	0/5	0/5	0/5		5.5-?	630+
		6	35	0/3	0/5	0/5		5.5-?	630+
		7	24	0/4	0/5	0/5		5.5-?	630+
		8	47	0/4	0/5	0/5		5.5-?	630+
		9	21	0/4	0/5	0/4		5.5-?	630+
	B	Rabbit control		5/5	5/5	1/5	1/5	7.7	—
		10	26	0/5	0/4	0/3		5.5-?	160+
		11	57	0/5	0/5	0/5		5.5-?	160+
		12	20	0/5	0/5	0/5		5.5-?	160+
		13	17	0/5	0/4	0/5		5.5-?	160+
		14	37	0/4	0/3	0/5		5.5-?	160+
		15	37	0/5	0/5	0/5		5.5-?	160+
		16	44	0/5	0/5	0/4		5.5-?	160+
		17	37	0/4	0/3	0/5		5.5-?	160+
		18	18	4/5	0/4	0/5		6.4	20
		19	27	0/5	0/4	0/5		5.5-?	160+
Shanghai	C	Rabbit control		5/5	5/5	3/5	0/5	8.2	—
		1	19	0/5	0/5	0/5		5.5-?	500+
		2	30	0/5	0/5	0/5		5.5-?	500+
		3	51	0/3	0/3	0/3		5.5-?	500+
		4	38	0/5	0/3	0/5		5.5-?	500+
		5	30	0/5	0/5	0/5		5.5-?	500+
	D	Rabbit control		5/5	5/5	1/5	0/4	7.6	—
		6	32	0/4	0/3	0/5		5.5-?	130+
		7	49	0/4	0/5	0/5		5.5-?	130+
		8	16	0/5	0/5	0/5		5.5-?	130+
		9	27	0/5	0/5	0/5		5.5-?	130+
		10	31	3/5	0/4	0/3		6.2-?	25+?
		11	18	0/5	0/5	0/5		5.5-?	130+
		12	20	0/3	0/5	0/5		5.5-?	130+
		13	32	4/5	0/3	0/5		6.4-?	16+?

a seasonal increase previously noted by others,[2,3] the total numbers are, nevertheless, small. While not all of the summer cases are necessarily caused by the virus of Japanese B encephalitis, it is probable that a good many of them may be. Since the majority of human beings obviously escape with inapparent infection, one should at least consider the possibility that the unknown factors which predispose to clinically apparent infection may be found less frequently among the Chinese than among the Japanese.

Summary. Complement fixation tests on acute and convalescent sera established the virus of Japanese B encephalitis as the cause of acute encephalitis in a Chinese physician in Shanghai during July, 1946. Evidence of extensive, *inapparent* dissemination of this virus among the populations of Shanghai and Tientsin, where no epidemics of encephalitis have been observed, was obtained when neutralizing antibodies for the Japanese B encephalitis virus were found in 85% of 13 Chinese residents of Shanghai, aged 16 to 51, and in 89% of 19 life-long, Chinese residents of Tientsin, aged 17 to 57, all without history of encephalitis. Since the great majority of human beings escape with inap-

TABLE III.
Cases of Acute Encephalitis Admitted to Central Hospital, Peiping (1942-1945).

	June, July, August, September				Other 8 months			
Year	No. admitted	No. diagnosis ?	No. died	Ages of patients yr	No. admitted	No. diagnosis ?	No. died	Ages of patients yr
1942	6	0	2	20, 18, 17, 26, 16, 19	4	1	2	?, 19, 10, 17
1943	6	1	3	42, 29, 23, 13, 21, 11	5	1	3	10, 36, 16, 29, 36
1944	12	3	5	16, 45, 65, 24, 8, 9, 25, 37, 47, 4, 22, 15	6	3	3	1, 40, 5, 3, 40, 2
1945	6	1	2	13, 50, 48, 14, 24, 15	2	1	0	13, 19

Data supplied by Dr. William H. L. Chung, Medical Director of Central Hospital.

parent infection even in Japan, where severe epidemics have occurred, the question is raised whether the unknown factors, which predispose to clinically apparent infection, may be found less frequently among the Chinese than among the Japanese.

15904

Electrophoretic Studies on the Leukocytosis-Promoting Factor of Exudates.[*]

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The studies of one of us (V.M.) have demonstrated the presence of a factor in inflammatory exudates of dogs capable of inducing both a growth in the bone marrow and a discharge into the circulation of polymorphonuclear leukocytes.[1] This canine material is active in human beings, suggesting its possible clinical application.[2] Salting out studies have indicated its close association with the pseudoglobulin fraction of exudates.[3] Recent studies at Temple University by one of us (V.M.) indicate that the active component of the leukocytosis-promoting factor (abbreviated as the LPF) appears to be a polypeptide. This has been shown by aging the LPF. The material splits as an active soluble polypeptide component from the rest of the molecule.[4] The present studies represent work performed in the past at Duke University by means of the Tiselius electrophoretic apparatus which indicates that the leukocytosis-promoting factor of exudates seems to be associated with the α_1 and α_2 globulins of exudates.

Materials and methods. The leukocytosis-promoting factor was obtained from exudate of dogs essentially as described by one of us.[2] Electrophoresis was carried out as previously described at 1°C in barbital buffer at pH 8.6 and 0.1 M ionic strength.[5,6] The patterns representing the migrating boundaries were recorded by the method of crossed slits described by Svensson.[7]

The serum was prepared for electrophoresis

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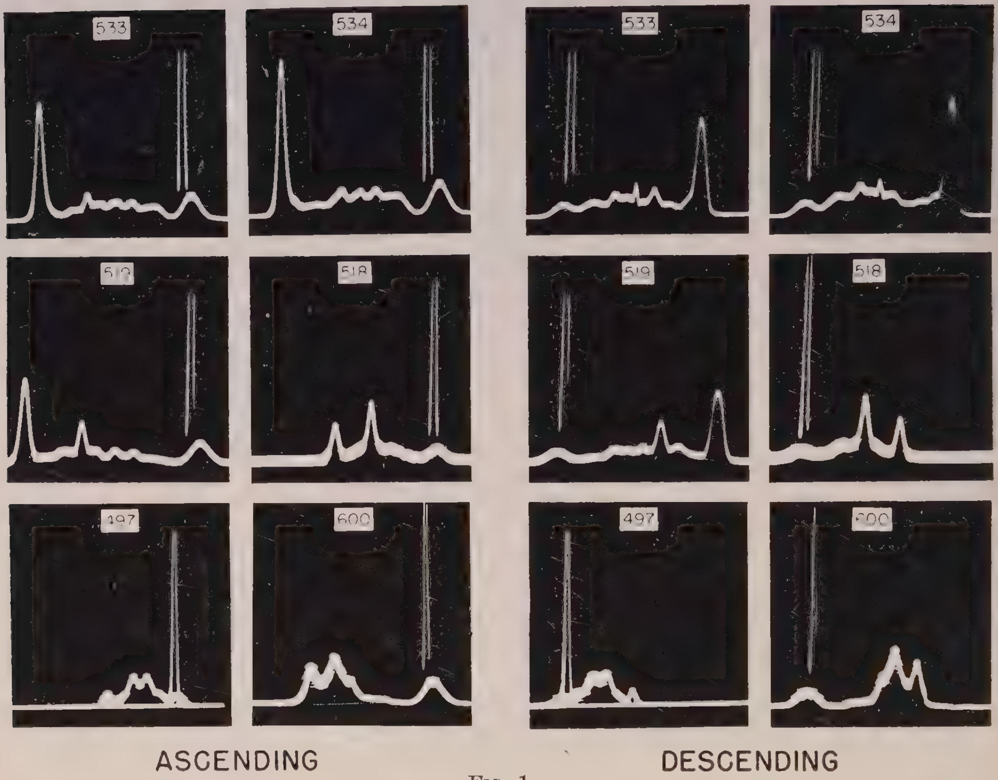

FIG. 1.

TABLE I.

This Table Gives the Mobilities and Percentage Composition of the Sera and Fractions Studied Electrophoretically. The Values Given for Normal Dog Serum Were Obtained from a Previous Study by Dr. G. R. Cooper.

Mobilities of Components $\times 10^{-5}$ cm/sec/volt

		A	α'_1	α_1	α'_2	α_2	β'	β	γ'	γ
Normal dog serum	T533	6.90	6.23	5.36	4.65	4.08	3.64	3.16	2.63	1.61
” ” ”	T534	6.81	6.15	5.38		4.10	3.52	3.11	2.64	1.68
Inflammatory exudate used for 14D fraction of 2-5-45	T519	6.89	5.97	5.24	4.54	3.87		3.18	2.53	1.67
LPF fraction of 14D of 2-5-45	T518	6.89	5.99	5.30	4.48			3.07	2.53	1.60
LPF run 6-4-45	T497	6.90		5.45	4.35			3.00		1.65
LPF 760 No. 12646 5-20-46	T600	6.85		5.45	4.50			3.01		

% Composition. Avg of Ascending and Descending Sides.

	A	α'_1	α_1	α'_2	α_2	β	γ'	γ
T533	42.0	10.3		15.0		11.8	9.6	11.3
T534	41.3	11.4		11.9		11.4	10.9	13.1
T519	41.7	3.7	9.4	19.4	7.6	6.8	7.8	3.6
T518	23.1	3.2	8.7	42.6		12.7	5.4	4.3
T497	9.2		7.6	40.6		31.1		11.6
T600	29.6		50.7	14.7		5.1		

by dilution with barbital buffer to approximately 2% protein concentration, and dialyzed against frequently changed buffer at 2° to 8°C. Protein concentrations were determined by falling-drop procedures[8] standardized by Kjeldahl analysis. Time and voltage are given in Fig. 1.

Component analyses were made in measurements in photographic enlargements of the electrophoretic diagrams in which the components were separated by vertical lines to the base at the minima between peaks.[9] The base line has been removed from the starting boundary by lowering the third blade of the angle slit, in those plates which show no base line. In the latter cases the base line is constructed by drawing lines connecting the portions of the base line remaining at the extremities of the patterns. The respective areas are bounded by the mid-line of the curve above, and the mid-line of the base below. The mobilities of the various components were calculated from the distances measured from the starting point of migration to the maximum ordinates of the respective peaks.[10] Calculations were made for both the ascending and the descending sides, and the values given in Table I are the average of the 2 sets of data.

Conclusions. The leukocytosis-promoting factor (LPF) of inflammatory exudates when studied electrophoretically in a Tiselius apparatus appears to be distributed primarily between the α_1 and α_2 globulins of exudates.

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15905

Effect of Feeding Potassium Acid Saccharate in the Diet of Rats for Successive Generations.*

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In former studies with various carbohydrates and their derivatives, calcium arabonate was fed to rats for long periods of time.[1,2] These compounds failed to affect deleteriously the rate of growth in 3 successive generations of rats. Recently potassium acid saccharate has been made available for experimental purposes and certain dietary uses. It was deemed advisable to conduct similar long-term feeding experiments with rats in which this compound was incorporated in their diet. A search of the literature failed to reveal any information on the fate of saccharic acid in the animal body.

Material and Methods. Potassium acid saccharate is a white crystalline powder having a pleasant acid taste. It is only slightly soluble in cold water but soluble in hot water. The compound employed in this investigation assayed not less than 99.5% purity and conformed to the heavy metals limit of 10 parts per million established by the United States Pharmacopoeia XII, p. 371, for potassium bitartrate. The compound was free of oxalic acid and gave a negative test for the nitrate ion. Various samples had neutralization equivalents ranging from 248.5 to 273.

The compound was added to a balanced diet of Purina Fox Chow in a concentration of 5%. The powder was mixed intimately with the food and fed to the animals. Special care was observed to insure the complete consumption of all the food. The rats were

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* The expense of this investigation was defrayed in part by a grant from the Sugar Research Foundation, Inc., of New York.

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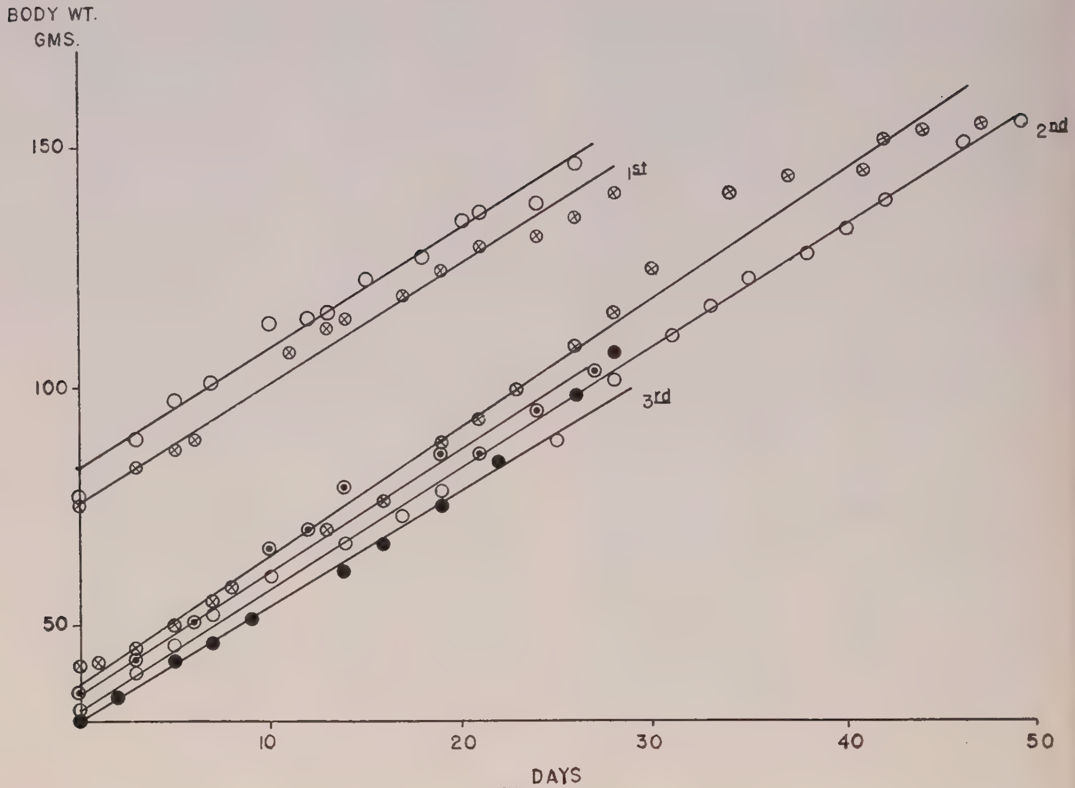

FIG. 1.

Growth curves of first, second, and third generation rats fed 5% potassium acid saccharate. ○ = experimental animals 1st and 2nd generation; x in ○ = 1st and 2nd generation control animals; dot in ○ = 3rd generation control animals; ● = 3rd generation experimental animals.

unable to select certain portions of their diet and reject the experimental compound. Water was allowed at all times. Greens were fed once weekly.

Fifteen young male and 15 young female rats were selected for this study. A similar grouping of 15 males and females comprised the control groups. The average weight of these animals was 76 g; the smallest weighed 73 and the largest 80 g. All animals were weighed on alternate days and males and females were kept separate. A growth curve of the body weights of the experimental and the control groups is given in Fig. 1. The food consumption of the animals fed the experimental diet was compared with the animals on the control diet at periods of time during the experiment. Both groups of animals consumed on the average approximately 10 g of food per rat per day, indicating that the presence of the potassium acid saccharate in their diet was not objectionable to the animals from the standpoint of taste. Periodically male and female animals were sacrificed for histologic examination of the viscera.

This experimental plan was followed with 2nd and 3rd generation rats. A summary of the data obtained is illustrated in the form of growth curves for the 3 generations compared with untreated animals (Fig. 1).

First Generation Rats. These animals produced 126 young in 71 days. The average weight of the young of rats fed the potassium acid saccharate diet, at the time of weaning (21 days) was 26 g. The average weight of the control animals at time of weaning was also 26 g. The control animals produced 99 young in a similar time period. Throughout this period the animals appeared in a good nutritional state. After 71 days the breeding was discontinued but the animals continued to receive the potassium acid saccharate in their food. At the termination of the experiment these animals were sacrificed and the organs subjected to microscopic examination.

Second Generation Rats. Twenty-five animals from each group were selected at the time of weaning and their growth rate recorded. The offspring of experimental animals continued to receive the potassium acid saccharate diet; the control animals received the basic diet. At this time males and females were separated. At the completion of the growth curve experiment the animals were mated. The remainder of the animals continued to receive the potassium salt in their food. All of these rats remained in an excellent nutritional state. These rats produced 78 offspring in 42 days; the control animals produced 73 young in a similar time period. The average weight of the young of both groups was approximately the same at the time of weaning.

Third Generation Rats. A similar grouping of the third generation animals was made and their growth rate recorded (Fig. 1). When these rats had attained a body weight of 150 g the experiment was terminated. All of these animals remained in excellent nutritional condition.

Histologic Examination of Viscera. A total of 47 animals were autopsied after receiving the 5% potassium acid saccharate diet for varying periods of time. Grossly, these animals exhibited no pathological lesions in any of the important viscera. Sections of the liver, kidney, spleen, duodenum, pancreas, heart, sternum, brain and lungs were prepared for microscopic examination after staining with hematoxylin and eosin. Histologically these sections appeared normal and compared favorably with similar sections prepared from 5 of the control animals. Five first-generation animals that received the potassium acid saccharate diet for approximately 7 months exhibited no abnormalities either grossly or histologically upon autopsy. It is estimated that each of these animals consumed approximately 105 g of potassium acid saccharate in their food throughout this time period.

Conclusions. The feeding of 5% potassium acid saccharate in the diet of the white rat for 3 generations does not significantly alter the growth pattern or fecundity. No significant pathological lesions were observed in the liver, kidney, intestinal tract, pancreas, heart, brain, bone, spleen or lungs of these animals. Subsequent experiments are planned to determine the fate of this compound in the animal body.

The potassium acid saccharate used in this investigation was kindly supplied by Dr. Louis Long, Jr., and Dr. Allen Scattergood of the Sugar Research Foundation, Inc., Laboratory of the Massachusetts Institute of Technology, Cambridge, Mass.

15906

Failure of the Liver of the Monkey to Inactivate Estrogens *in vivo*.*

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On the strength of numerous reports that the liver inactivates estrogens or prevents their reaching the systemic circulation in the rat,[1-8] guinea pig,[9,10] rabbit,[5,11] and dog,[12-14] it is often presumed that this is a universal hepatic action. For example, the testicular atrophy, gynecomastia, and changes in hair distribution frequently seen in men with cirrhosis of the liver,[15-17] the elevated level of urinary estrogens in men with infectious hepatitis[18] and hepatic cirrhosis,[19] as well as menstrual disturbances[20] and poor post-partum involution of the uterus[21] which may be relieved by treatment with the vitamin B complex have all been attributed to the failure of the liver in these patients to inactivate estrogens. As compared with that of the rat, however, human liver is reported[22] to exhibit much less capacity to inactivate estrogens *in vitro*, and the ability of the liver to inactivate estrogens *in vivo* has apparently not been examined directly in man or any of the primates. Accordingly, the capacity of the liver of the monkey to inactivate estrogens *in vivo* has been tested by implanting pellets in the spleen as has been done in lower animals. Since the venous drainage of the spleen is into the liver, estrogen absorbed from intrasplenic pellets is exposed to the action of the liver before reaching its target organs and, in the animals listed above, exerted none of its characteristic actions.

Five ovariectomized monkeys, *Macacus rhesus*, were employed. The diet consisted of Purina "Laboratory Chow" fed *ad libitum* and supplemented twice weekly with cabbage. On this regime the animals grow well and reproduce. At the end of a preliminary period of at least 2 weeks, during which daily vaginal smears stained by the method of de

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Allende, Shorr, and Hartman[23] were examined and the condition of the sex skin was recorded daily, a compressed pellet of estrogen was introduced into the spleen in a subcapsular position. In 2 animals the estrogen was estrone and in 2 animals estradiol. The pellets of estrone weighed 4.0 and 9.0 mg, the pellets of estradiol weighed 4.0 and 11.0 mg. In the hope of relieving any dietary deficiency in the vitamin B complex, which is reported[24] to decrease inactivation of estrogen by the rat's liver, 2 animals were daily given subcutaneously an aqueous solution of members of the B complex for a period of 10 days preceding the implantation of the pellet of estrogen and throughout the interval that the pellet (in one animal estrone and in the other estradiol) was in place. The daily supplement was 1 mg thiamine hydrochloride, 5 mg nicotinic acid, 1 mg riboflavin, 1 mg pyridoxine hydrochloride, 3 mg calcium pantothenate, 50 mg choline chloride.[25] Instead of an intrasplenic pellet of estrogen, the 5th monkey was given a daily intramuscular injection of 16.6 μg of estradiol benzoate dissolved in sesame oil.

The 5 animals responded in essentially the same manner. The sex skin reddened to the same extent and with the same promptness in the animals bearing intrasplenic pellets as in the animal given intramuscular injections. The vaginal smears prior to treatment in all of the animals were typically those of ovariectomized monkeys in being scanty and composed of leucocytes and detritus. Within 3 days after the introduction of the intrasplenic pellet of estrogen the vaginal smear had increased in amount and within 5 days was composed almost or entirely of cornified epithelial cells of types IIIb and IV.[23] The identity of the estrogen or the size of the pellet made no obvious difference in the intensity and promptness of either of the 2 responses, nor were the responses to the intrasplenic pellets in any way different from those of the animal given subcutaneous injections. The administration of the B vitamins was without evident influence.

After 9, 15 or 18 days the pellets were removed. Two animals were thereafter given no further treatment; each exhibited a typical estrogen-deprivation menstruation of 5 days' duration beginning 7 days after removal of the pellet. The other 2 animals were given a daily intramuscular injection of progesterone beginning on the day of removal of the pellet. The daily dose of progesterone in one animal was 0.5 mg, an amount that fails to inhibit estrogen-deprivation bleeding indefinitely.[26] This animal began menstruating 15 days later. The second animal was given 1.0 mg of progesterone daily for 20 days at which time a uterine biopsy was taken; 3 days after the last injection of progesterone menstruation started. The animal given the intramuscular injections of estradiol benzoate was so treated for 12 days; thereafter a daily intramuscular injection of 1.0 mg of progesterone was substituted. A uterine biopsy was taken on the 23rd day of the latter course of injections. The 2 biopsy specimens were alike in exhibiting a premenstrual endometrium with the typical glandular change, moderate edema, and large, vesicular stromal nuclei with distinct nucleoli and large chromatin particles.[27]

Inasmuch as the failure of the liver to inactivate the estrogen absorbed from the intrasplenic pellets might have been the result of deficient liver function (despite the administration of B vitamins to 2 of the animals), hepatic function was examined at the time of removal of the pellets by the bromsulphalein test[28] and the cephalin-cholesterol flocculation test.[29] The latter test was negative at 24 and 48 hours. The retention of bromsulphalein 30 minutes after the intravenous injection of 5 mg per kg was between 2% and 4%, normal values for the monkey. A second possibility that could have accounted for the failure of inactivation was accessory venous drainage of the spleen by one or more routes that circumvented the liver. This possibility was examined in 2 of the animals at the time of removal of the pellet by injection 0.25 cc of bromsulphalein (50 mg/cc) into the spleen after clamping the splenic vein. Five minutes later a sample of peripheral venous blood was drawn and tested for the dye. None was found.

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Clearly, sufficient amounts of estrogen passed from the spleen into the general circulation to elicit 4 of the characteristic responses ordinarily evoked by estrogens in the ovariectomized monkey: reddening of the sex skin, modification of the vaginal smear, growth and edema of the endometrium, and estrogen-deprivation menstruation. Moreover, the estrogen was absorbed from intrasplenic pellets of a size that provoke no responses in ovariectomized rats. In at least 2 of the animals venous drainage of the spleen by routes other than that through the liver was inadequate to transport detectable amounts of dye into the general circulation. It seems reasonable to presume, therefore, that the estrogen absorbed from the intrasplenic pellets passed through the liver. It is not known, of course, whether derangement of the ability of the liver to prevent estrogen's reaching the general circulation would exist in the presence of normal liver function as judged by other tests. In any case, the administration of B vitamins did not modify the responses given to the estrogen absorbed from the intrasplenic pellet.

It does not follow from these observations that the monkey's liver is unable to inactivate estrogens *in vivo*. It is apparent, however, that estrone and estradiol were not inactivated completely. Since, on the other hand, the liver of the rat completely inactivates estrogens absorbed from intrasplenic pellets (or prevents their reaching the general circulation), it seems evident that the monkey's liver exhibits less, perhaps much less, ability to inactivate estrone and estradiol. This finding is consistent with the report[22] of slight inactivation of estrogen by human liver *in vitro* as compared with the liver of the rat.

Summary. Intrasplenic implantation of pellets of estrone and estradiol in 4 ovariectomized monkeys was followed by reddening of the sex skin and alteration of the vaginal smear as promptly and with the same intensity as in a control animal given estradiol benzoate intramuscularly. Endometrial changes in the animals were alike, and removal of the intrasplenic pellet was followed by typical estrogen-deprivation menstruation. Liver function tests revealed no hepatic damage and administration of B vitamins did not reduce the activity of the intrasplenic estrogens.

15907

Effects of DCA on Digitoxin Toxicity in Cats.

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It has been noted that a number of patients with Addison's disease, while apparently being satisfactorily treated with NaCl added to the diet and supplementary DCA (desoxycorticosterone acetate), developed rather unusual cardiac irregularities, which in some cases have terminated rapidly in death. These deaths, apparently cardiac in origin, have appeared to be associated with an overactive vagus,[1] and have also resembled those following an overdosage of a digitalis glycoside.

Addisonian patients have been suspected of being hypersensitive to vagal stimuli, a suspicion which has been strengthened by the experiments of Perera,[2] who demonstrated markedly elevated responses of these patients to small doses of Mecholyl. Pharmacological analysis of the action of the digitalis glycosides has revealed them to have effects directly upon the myocardium and myocardial conduction system and to increase the heart's sensitivity to local and central vagal stimulation. Other studies, stimulated by the empirical observation of a fairly consistent bradycardia in patients with obstructive jaundice, have shown certain of the bile salts to be capable of producing cardiac irregularities and death by both vagal and direct actions upon the heart.[3,4]

In view of the structural resemblance of the adrenal cortical steroids (DCA in particular) to the digitalis glycosides, and the bile salts, it was considered desirable to investigate the possibility that DCA might potentiate the toxic effects of a digitalis glycoside.

Methods. In order to study this effect, 2 groups of healthy cats (8 cats in each group) were selected, weighing between 2 and 4 kg. The cats in Group 1 received 10 mg of DCA* in sesame oil, by subcutaneous injection, every day for 10 days (100 mg in all)—half of this group was on a normal diet, half on a diet containing about 2 g additional NaCl daily. The cats in Group 2 received no DCA, but were similarly divided as to normal and high salt diets. On the 10th day of DCA administration, the DCA-treated animals and the control animals were subjected to the standard U.S.P. digitalis assay—using a pure glycoside, digitoxin.† The assay of the DCA-treated animals was performed between 3 and 6 hours after administration of the final injection of 10 mg of DCA. The cats receiving DCA seemed to be abnormally susceptible to serious central respiratory depression by ether, and 2 animals of this group died during the induction of anesthesia. The number of doses of digitoxin (0.0025%, 1 cc/kg being injected rapidly intravenously at 5-minute intervals) and the total time required for the onset of ventricular fibrillation were recorded for all animals. EKG tracings were made in all the animals (with an initial control record and subsequent tracings at 15-minute intervals). Continuous observation of the EKG pattern was made possible by the use of the Sanborn cardioscope. Immediately following the onset of ventricular fibrillation, blood was taken under oil from the inferior vena cava for direct Na and K determinations. The adrenal glands were weighed, and histological sections were made from samples of heart, kidney, and adrenal.

Results. There was no appreciable difference in the total lethal dose or in the time required for onset of ventricular fibrillation between the DCA-treated animals and the control group (Table I). There was no apparent effect of the high salt diet on the final results within the groups. The variations in the values for the direct Na and K determinations were so great that no significant elevation in Na or lowering in K could be demonstrated in the serum of the DCA-treated animals. Furthermore, the histological sections of the tissues of the DCA-treated animals did not show any consistent alterations from the control group. The EKG tracings varied widely, but were similar in the 2 groups; also, no difference could be noted by observing the continuous cardioscope tracing of each cat. The average weight of the paired adrenal glands was 542 mg for 5 cats of the control group and 393 mg for 6 cats of the group receiving DCA. The significance of the difference in the means, calculated by Fisher's method of computing t, was represented by $P = 0.05$. There was no

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† Digitoxin (Lilly Research Lab. No. 405741-A) was kindly donated by Dr. K. K. Chen, of the Lilly Research Laboratories.

TABLE I.
Lethal Effect of Digitoxin Assayed by Method of U.S.P. XII.

		Control animals			DCA-treated animals	
	No.	Lethal dose of 0.0025% digitoxin, cc/kg	Total assay time, min	No.	Lethal dose of 0.0025% digitoxin, cc/kg	Total assay time, min
Normal diet	1	10	45	A	12	50
	2	12	55	B	12	55
	3	15	70	C		
	4	13	66	D	13	62
		—	—		—	—
Avg		12.5	59		12.3	55.7
High salt	5	15	72	E	11	54
	6	11	53	F		
	7	13	62	G	15	73
	8	13	63	H	12	55
		—	—		—	—
Avg		13.0	62.5		12.7	60.7
Avg of combined groups		12.75	60.75		12.5	58.2

significant difference between average adrenal weights if these were expressed as percentages of the body weights $(P = <0.2$ but $>0.1)$.

Conclusion. These observations failed to support the hypothesis that DCA potentiates the toxic effects of digitoxin.

15908

Relation of Adrenal Cortex to Serum Peptidase Activity.*

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Attention has been drawn by several investigators[1-3] to the rapid hydrolysis of *l*-leucylglycylglycine (LGG) by the sera of rabbits, swine, horses, rats and humans. The splitting of LGG was attributed by Grassmann and Heyde[1] to the action of a serum "aminopolypeptidase," but a recent study[4] has presented evidence for the participation of at least 2 enzymes in the hydrolysis of this substrate by rabbit serum, and presumably by other sera as well. One of these enzymes has been identified as a manganese-activatable leucine aminopeptidase, for which *l*-leucinamide acetate (LA) is a satisfactory substrate. The other serum enzyme which splits LGG is a peptidase with a different, but hitherto undefined, specificity. Both of these enzymes are closely related in several properties to LGG-splitting enzymes found in extracts of skin and lung,[4] intestinal mucosa,[5] muscle,[6] and in leucocytes and lymphocytes.[7] In view of the widespread distribution, in

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† James Hudson Brown Junior Fellow 1946-1947.

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TABLE I.

Hydrolysis of Leucylglycylglycine (LGG) and Leucinamide (LA) by Mouse Serum

				Hydrolysis			
				No activator added		0.001 M $MnSO_4$ present	
Substrate	cc of mouse serum per cc test solution	pH	Time in min	%*	K°	%*	K°
LGG	0.2	8.0	15	13	0.87		
			30	26	0.87		
			45	35	0.78		
			60	45	0.75		
	0.1	8.0	15	6	0.40		
			30	13	0.43	36	1.20
			45	20	0.45		
			60	27	0.45	66	1.10
LA	0.16	8.2	30	24	0.80	56	1.87
			60	47	0.78		
			90	69	0.77		
	0.12	8 3	30	16	0.53	45	1.50
			60	36	0.60	82	1.37
			90	54	0.60		
	0.08	8.0	30	12	0.40	31	1.03
			60	23	0.38	61	1.02
			90	38	0.42		

* % of the hydrolysis expected on the complete splitting of one peptide linkage.

tissues, of similar proteolytic enzymes which act on LGG, the possibility has been considered[4] that these enzymes have a common origin in ubiquitous cells such as lymphocytes. Furthermore, the suggestion has been made that the presence of the LGG-splitting peptidases in the sera of all animals tested might be due to the liberation of these enzymes into the circulation in the course of the disintegration of lymphoid tissue. In previous studies,[8] it was shown that the rate of dissolution of lymphoid tissue is controlled by the hormonal secretion of the adrenal cortex. The experiments reported in the present communication support the view that the serum peptidases which act on LGG arise, to a significant degree, from the turnover of lymphoid tissue, since the administration, to mice, of adrenal cortical extract or of pituitary adrenotrophic hormone, results in a marked increase in the peptidase activity of the sera of these animals.

Methods. Male mice (7-14 weeks old) of the CBA strain (Strong) were used. Water and Purina Fox Chow were available to the animals at all times. The diet was supplemented by a mixture consisting of 91% calf meal and 3% each of cod liver oil, dried yeast powder and wheat germ. Blood was obtained by heart puncture and pooled sera were used for the determination of enzyme activity. In the enzyme experiments, the substrate concentration was 0.05 mM per cc of the test solution, the pH was maintained near pH 8 with 0.02 M veronal buffer, and the temperature was kept at 38°. The rate of proteolytic action was followed by the titration method of Grassmann and Heyde.[9] The synthesis of the substrates LGG and LA is described elsewhere.[10,11] The hormone preparations employed in this study were an aqueous extract of adrenal cortex (Wilson), adrenal cortical steroids in oil (Upjohn), and hog

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TABLE II.
Effect of Hormones on Serum Peptidase Level of Mice.

Treatment of mice	Substrate	No. of mice	No. of serum pools	K°*	% increase	"P"†
None	LGG	24	12	0.42 ± 0.09		
	LA	22	10	0.38 ± 0.22		
				0.86 ± 0.28‡		
Aqueous adrenal cortical extr. (1 cc) inj. 3-3.5 hr before bleeding	LGG	6	4	0.93 ± 0.07	120	<0.01
Cortical steroids in oil (0.1 cc) inj. 5-5.5 hr before bleeding	LGG	8	4	1.42 ± 0.42	235	<0.01
Cortical steroids in oil (0.1 cc) inj. 9-9.5 hr before bleeding	LGG	8	7	1.61 ± 0.56	272	<0.01
	LA	12	12	0.83 ± 0.56	126	0.038
				1.93 ± 0.87‡	136	0.018
Adrenotrophic hormone (1 mg) inj. 9-10 hr before bleeding	LGG	8	8	1.24 ± 0.17	188	<0.01
	LA	6	6	0.61 ± 0.43	64	0.3
				1.45 ± 0.84‡	69	0.1

* Mean value and standard deviation for 0.1 cc of serum per cc of test solution.
† Calculated according to "Student's" t method of Fisher.[13]
‡ Determined in the presence of 0.001 M $MnSO_4$.

pituitary adrenotrophic hormone[12] (2 mg per cc of aqueous solution). These preparations were administered by subcutaneous injection.

Hydrolysis of LGG and LA by Mouse Serum. The data in Table I show that LGG is hydrolyzed extremely rapidly by mouse serum. As was noted previously for rabbit serum,[4] the initial rate of hydrolysis accords with the kinetics of a zero order reaction. The rate may be expressed therefore by the constant K°_{LGG} which is defined as per cent hydrolysis per minute. Within the limits of enzyme concentration given in Table I, the value of the constant is proportional to the amount of serum per cc of the test solution.

For a series of 24 mice, from which there were obtained 12 samples of pooled sera, the value of K°_{LGG} was found to be 0.42 ± 0.09 for 0.1 cc of mouse serum per cc of test solution (*cf.* Table II). This value is much greater than that observed for rabbit serum (0.18) or for human serum (0.035).[4]

It will be noted from Table I that the addition of 0.001 M $MnSO_4$ greatly accelerates the hydrolysis of LGG by mouse serum, thus indicating the presence of appreciable amounts of leucine aminopeptidase. This observation is similar to the result obtained with guinea pig serum,[2,4] but is in contrast to the behavior of rabbit and horse sera which do not appear to be rich in aminopeptidase activity.

The conclusion that a large part of the hydrolysis of LGG by mouse serum is due to a manganese-activatable leucine aminopeptidase is confirmed by the finding that LA is split rapidly by this serum, and that the rate of hydrolysis is greatly increased by the addition of $MnSO_4$ (Table I). As in the case of the hydrolysis of LGG, the initial rate of enzymatic action follows the kinetics of a zero order reaction, and the rate may be expressed, therefore, by the constant K°_{LA}. It will be noted that the variation in the capacity of various mouse sera to split LA is much greater than in the case of LGG (*cf.* Table II).

Effect of Administration of Hormones on the Serum Peptidase Level. The data in Table II show that, following the subcutaneous injection of adrenal cortical extracts or of adrenotrophic hormone, there occurs an appreciable rise in the value for K°_{LGG} per unit volume of serum. It is of interest that the most marked effect is observed about 9 hours after the administration of adrenal cortical steroids in oil. Previous studies have shown that, in mice, the degree of lymphocyte dissolution,[8] and resultant blood lymphopenia,[14] are maximal 6 to 9 hours after the injection of this hormone preparation. During this period, there is also an increased rate of release of protein from lymphoid tissue to the circulation.[15]

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Following injection of adrenal cortical steroids in oil, the increase in activity toward LGG is accompanied by an increase in the rate of hydrolysis of LA. The latter result was not found to be as reproducible as was the effect on the hydrolysis of LGG, but the difference in aminopeptidase activity for the sera of treated and untreated mice appears to be statistically significant. On the other hand, the injection of adrenotrophic hormone did not cause a significant rise in activity toward LA. This result may be due to the small number of serum samples tested.

It is of interest that the addition of 0.001 M $MnSO_4$ results in an activation of the hydrolysis of LA by sera from injected animals. This suggests that the increase in the activity toward LA, as well as towards LGG, following the administration of hormone, is due to a mechanism other than an increase in a serum constituent with a mode of action similar to that of $MnSO_4$. Although the possibility cannot as yet be excluded that the injection of hormone releases an enzyme activator of unknown nature, or removes a substance which is an inhibitor, the conclusion seems justified that there occurs an actual increase in the amount of circulating peptidases. That this increase cannot be accounted for by hemoconcentration resulting from hormone administration is shown by the data for the protein content of the sera of normal and treated mice. Determinations of the total nitrogen content of a series of untreated mice gave a value of 10.5±1.5 mg per cc, while a comparable group of sera from mice treated with aqueous adrenal cortical extract showed a nitrogen content of 9.9±1.2 mg per cc. In the case of mice which had received adrenal cortical steroids in oil, the nitrogen content of the sera was 11.8±1.8 mg per cc, and for mice treated with adrenotrophic hormone, the serum nitrogen was found to be 10.3±1.2 mg per cc.

It may be added that preliminary studies on rabbits have shown that the injection of cortical steroids in oil or of adrenotrophic hormone also produces a significant increase in the capacity of the sera of these animals to hydrolyze LGG. The effect observed in rabbits, however, does not appear to be as marked as that noted for mice.

Discussion. The control of the serum peptidase level by the hormonal secretion of the adrenal cortex may aid in explaining increases in serum proteolytic activity observed following a nonspecific stimulus known to augment pituitary-adrenal cortical secretion. Thus, an abrupt rise in the serum peptidases of the dog, cat, rat and calf was reported[6] to result from the burning of a superficial skin area. This type of injury is a powerful activator of the pituitary-adrenal cortical mechanism.[16] It may be noted also that sera from patients with fever have been found[1] to exhibit a greater activity toward LGG than that observed for sera from normal humans.

In addition, the data in the present investigation focus attention on the proteolytic enzymes of lymphoid tissue. It has been found[17] that extracts of calf thymus are unusually rich in enzymatic activity toward LGG and that the enzyme specific for this substrate may readily be separated from the accompanying manganese-activatable leucine aminopeptidase which hydrolyzes LA. Calf thymus, therefore, is a suitable starting material for the purification of the LGG-splitting peptidase preparatory to a study of the specificity and physiological effects of this enzyme.

Summary. A single, subcutaneous injection, in mice, of adrenal cortical extracts or of pituitary adrenotrophic hormone causes an appreciable rise in the serum peptidase level of these animals. It has been suggested that this increase in enzyme activity is a result of the acceleration, by the hormones of the adrenal cortex, of the rate of turnover of lymphoid tissue.

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15909

Studies on Cigarette Smoke Irritation. II. The Role of Nicotine.

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That nicotine in the form of its free base is subjectively quite irritating has been known for many years. Thus Vandencorput[1] stated that the least touch of this substance in the mouth caused a burning sensation. Shortly thereafter, Braun[2] noted that bringing a drop of a solution containing one drop of nicotine per dram of water into contact with the eye of a rabbit seemed very painful to the animal. Similarly, Dworzak and Heinrich[3] following self-experimentation with nicotine doses varying in amount from 1/32 drop to 1/16 drop in one dram of water, recorded that the smaller doses occasioned a burning sensation in the tongue, a hot, acrid irritation in the fauces, and when doses somewhat larger were used, the entire length of the esophagus felt as if it had been scraped by an iron instrument.

Hypodermically-injected nicotine also produces smarting at the site of injection,[4] and inhaled nicotine vapors produce a "scratchy" sensation on the mucous membrane of the mouth and throat.[5]

Bogen[6] explains this irritation action of nicotine alkaloid on the basis of the caustic effect of its alkalinity, and Mulinos and Osborne[7] and Weatherby[8] observed that neutral solutions of nicotine were nonirritating.

Recently,[9] we described a method for the quantitative evaluation of cigarette smoke irritation, as judged by its edema-producing properties, based on gravimetric determination of the increase in moisture content of the upper palpebral conjunctiva of the rabbit eye following its exposure to cigarette smoke. For clarity of subsequent presentation of results a brief description of the method follows:

Using a smoking machine of the type described by Bradford, Harlan and Hanmer,[10] which draws a 35 ml puff once per minute, 3 puffs are directed through a suitable eye cup placed over the widely opened eye of a morphinized rabbit. When one eye only of each rabbit is exposed, the other serving as a control, we have named the technic "Procedure A." When both eyes of each rabbit are exposed, thereby permitting comparison of 2 cigarettes on the same animal, the technic is called "Procedure B." In both procedures, following exposure to the smoke, a one-hour period is allowed for edema formation. The rabbit is then sacrificed, the upper lid membrane of each eye excised, weighed, dried in an oven to constant weight, and reweighed. From the wet and dry weights the ratio of moisture to dry weight is calculated and, in the case of Procedure A, the per cent of increase in water in the exposed membrane over that in the control membrane is determined. In a series of such determinations involving 2 or more groups of cigarettes, the differences in the ratios between groups are evaluated statistically for significance by analysis of variance.

In the present study we have applied these procedures to an evaluation of the role of nicotine in cigarette smoke irritation, and, by

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[1] Vandencorput, Ed., *Gaz. d. Hop.*, July 1, 1851; abstr. in *Am. J. Med. Sci.*, 1852, **23**, 552.

[2] Braun, H., *Arch. f. Ophthalmol.*, 1859, **5**, 112.

[3] Dworzak and Heinrich, *Mat. Med. d. rein. chem. Pflanzenstoffe*, p. 234; quoted by Hare, H. A., *Fisk Fund Prize Dissertation*, 1885.

[4] Johnston, L. M., *Lancet*, 1942, **2**, 742.

[5] Biederbeck, J., *Inaugural Dissertation*, Wurzburg, F. Staudenraus, 24 pp., 1908.

[6] Bogen, E., *Calif. and West. Med.*, 1936, **45**, 342.

[7] Mulinos, M. G., and Osborne, R. L., Proc. Soc. Exp. Biol. and Med., 1934, **32**, 241.

[8] Weatherby, J. H., *J. Lab. and Clin. Med.*, 1939-40, **25**, 1199.

[9] Finnegan, J. K., Fordham, Doris, Larson, P. S., and Haag, H. B., *J. Pharm. and Exp. Therap.*, 1947, **89**, 115.

[10] Bradford, J., Harlan, W., and Hanmer, H. R., *Ind. Eng. Chem.*, 1936, **28**, 836.

TABLE I.
Effect of Increasing Nicotine Content of Tobacco on the Edema-Producing Properties of Its Smoke.

Cigarette Lot No.	Nicotine content of tobacco (%)	Nicotine content of smoke per cigarette* (mg)	No. of observations	Mean of ratio differences†	Mean of % increase in moisture
1	0.50	0.55	20	2.07	42.28
2	1.02	1.28	20	2.11	47.92
3	1.73	1.83	20	2.46	52.09
4	2.15	2.47	20	2.32	47.96
5	2.62	3.01	20	2.09	41.72

* Represents nicotine content of main stream smoke (that which passes through the mouth end of the cigarette). For these nicotine analyses, two-thirds of the total length of the cigarette was smoked at a rate of one 35 ml puff of 2 seconds duration per minute.[12]

† Ratios for exposed tissues minus ratios for control tissues.

TABLE II.
Results of Tests for Edema-Production by Nicotine Alkaloid.

Nicotine concentrations (M)	No. of observations	Mean of ratio differences*	Mean of % change in moisture
0.001	10	—0.20	—4.75
0.01	10	—0.06	—2.02
0.1	10	—0.15	—3.03

* Ratios for exposed tissues minus ratios for control tissues.

modification of the mode of administration, to an evaluation of the irritant action of water solutions of nicotine.

Experimental. Two sets of experiments were performed. In the first of these, a single batch of Burley tobacco,* naturally low in nicotine (ca 0.5%), was divided into 5 lots. One lot was untreated and the remaining 4 were treated successively with preselected increasing amounts of nicotine malate solution. Subsequent analyses by the AOAC method[11] showed that after this treatment the 5 lots ranged in nicotine content from about 0.5 to 2.5% in increments of approximately 0.5% (Table I).

For the irritation tests 20 cigarettes from each lot were selected on the basis of uniform weight (±5%), brought to uniform moisture content (11.5-12.5%) by exposure to an atmosphere of suitable humidity, and their smoke analyzed for edema-production by Procedure A. The results are summarized in Table I.

In the second set of experiments, the edema-producing properties of nicotine alkaloid (0.001, 0.01 and 0.1 M in a 0.9% sodium chloride solution) were tested by Procedure A modified to the extent that 3 drops of solution instead of 3 puffs of smoke were held in contact with the tissues of the eye for a period of 3 minutes. Ten rabbits were used at each of the above 3 concentrations of nicotine. The results are summarized in Table II.

Discussion. Statistical analysis of the results summarized in Table I show that no significant differences exist between the edema-producing properties of the smoke from the 5 lots of cigarettes tested. It may be noted that the greatest mean difference in moisture to dry weight ratios resulted between the tissues exposed to lots 1 and 3 and 3 and 5, yet the probability of encountering a more divergent sampling was .45 and .51 respectively. Therefore, on the basis of these studies, it may be concluded that neither nicotine nor its combustion products contribute significantly *per se* to the edema-producing irritants in cigarette smoke.

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* Kindly supplied to us by Dr. W. D. Valleau, University of Kentucky Agricultural Experiment Station, Lexington, Ky.

[11] Assn. Official Agri. Chem., *Official and Tentative Methods of Analysis*, 5th ed., p. 64, 1940.

[12] Bradford, J. A., Harlow, E. S., Harlan, W. R., and Hanmer, H. R., *Ind. Eng. Chem.*, 1937, **29**, 45.

This conclusion does not hold for the subjective sensations of irritation produced by cigarette smoke. In blind tests, the average smoker had no difficulty in arranging these cigarettes in the order of increasing nicotine content on the basis of increasing irritation of the smoke.

The results obtained with nicotine solutions were quite similar. As seen in Table II, edema failed to result at any of the nicotine concentrations used. Tests at higher concentrations were not completed owing to the appearance of rather severe symptoms of systemic nicotine poisoning.

Again the lack of edema production bore no relation to the evidence of subjective irritation. Even though the rabbits were well depressed by morphine, the struggling that followed application of the higher concentrations of nicotine indicated a severe subjective irritation far exceeding that which in our experience results from application of smoke in amounts sufficient to produce massive edemas.

This lack of edema formation following nicotine application brings to mind the observation by Bardy[13] that local application of nicotine to the eye of the rabbit will block further production of conjunctivitis on administration of mustard oil. From this and other evidence, Bardy arrived at the conclusion that conjunctivitis produced by mustard oil was reflex in character and that nicotine interrupts the reflex path through acting on peripheral synapses. Carrying this argument further could lead to the postulate that nicotine tends in this manner to inhibit its own production of conjunctivitis. However this may be, it seems certain that while nicotine may markedly influence the subjective sensations of irritation from cigarette smoke it does not significantly contribute *per se* to its edema-producing properties.

Conclusions. Neither nicotine nor its products of combustion contribute significantly *per se* to the edema-producing properties of cigarette smoke, although it may definitely increase the subjective sensations of irritation.

We are indebted to Mrs. Sarah Guerry for technical assistance in performing the reported experiments.

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[13] Bardy, H., *Skand. Arch. Physiol.*, 1915, **32**, 198.

15910

Studies on "Vitamin P." I. Topically Applied "Vitamin P"-like Substances on the Mammalian Capillary Bed.*

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Since the introduction of the concept of "vitamin P" by Szent-Györgyi and his collaborators,[1] an extensive and controversial literature has appeared on the subject.[2] Recently, interest has been reawakened in the subject by the observations of Griffith, Couch and Lindauer[3] that rutin, a flavonol glycoside, decreases capillary fragility in hypertensive patients; and by the work of Lavollay, Par-

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† Present address: The Scripps Metabolic Clinic, La Jolla, Calif.

[1] Armentano, L., Bensáth, A., Bères, T., Rusznyak, I., and Szent-Györgyi, A., *Dtsch. med. Wchnschr.*, 1936, **62**, 1326.

[2] Anon., "Bibliography of Vitamin P" (256 refs.), *Nutrition Research*, 1945, **5**, No. 1 and 2.

[3] Griffith, J. Q., Jr., Couch, J. F., and Lindauer, M. A., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 228.

rot and their co-workers[4-9] who postulate that "vitamin P" exerts its various antihemorrhagic effects by the inhibition of oxidation of circulating epinephrine or adrenochrome, which then is capable of decreasing capillary fragility, "capillary permeability,"‡ and bleeding time, through a vasoconstrictive or tonic action on precapillary blood vessels.

In studying the direct or indirect effects of "vitamin P" on the filtration of fluid through the capillary wall, it is of importance to first describe possible effects on the vasomotion of the small blood vessels, since capillary blood pressure and capillary diameter may influence fluid filtration through the capillary walls. A few studies indirectly bearing on this subject have been reported,[11-13] in which vasoconstriction was observed in Laewen-Trendelenburg preparations of amphibian and mammalian extremities perfused with quercetin, quercitrin, rutin, myricetin and a quercetin diglucoside. One group of workers, however, reported dilatation by quercetin.[14]

In the light of recent concepts of the epinephrine-sparing action of "vitamin P,"[9,15,16] it became of interest to determine if several compounds claimed to have "vitamin P" activity, would show any microscopically observable vasomotor effects on the *intact* mammalian capillary bed when applied topically in the absence of extrinsic epinephrine.

Methods and Materials. The following substances were selected as representative compounds of the "vitamin P" group: rutin, sodium rutin acid succinate, sodium rutin acid phthalate, hesperidin, sodium hesperidin acid succinate, sodium hesperidin acid phthalate, methylated hesperidin chalcone, esculin ("vitamin P_2"),[17] *d*-catechin, epimerized *d*-catechin ("vitamin P_1"),[17] and *l*-epicatechin. In addition it was thought of interest to test "catechin red" (catechin tannin, catechin anhydride, "antivitamin P")[18] and adrenochrome.

The rutin was obtained from Dr. J. F. Couch, U. S. Department of Agriculture, Eastern Regional Laboratory, Philadelphia, and Dr. George Hazel, Abbott Laboratories, North Chicago. The hesperidin and rutin acid phthalates and succinates were synthesized by methods developed from suggestions contained in a Department of Commerce report on the activities of the I. G. Farbenindustrie,[19] for example:

Rutin Acid Succinate. A solution of 4.0 g rutin and 6.0 g succinic anhydride in 25 cc dry pyridine was heated 4 hours on a steam bath. After removal of the pyridine *in vacuo*, the residue was dissolved in 5% $NaHCO_3$, poured into dilute acetic acid, and the resulting clear solution saturated with ammonium sulfate and extracted with isopropanol. Re-

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[4] Parrot, J., *Gaz. méd.*, 1946, **53**, 157.

[5] Galmiche, P., *La rèsistance et la perméabilite des vaisseaux capillaires et vitamine P*, 1 vol., 144 pp., Le Francois, Paris, 1945.

[6] Lavollay, J., *L'autoxydation des diphénols, en particulier de l'adrénaline. Etat actuel du problème de la structure et du rôle fonctionnel de la vitamine P*, 1 vol., 138 pp., Hermann & Cie, Paris, 1943.

[7] Parrot, J., and Galmiche, P., *Le bull. méd.*, 1945, **59**, 413.

[8] Paraf, A., *La presse méd.*, 1944, **52**, 134.

[9] Javillier, M., and Lavollay, J., *Helv. Chim. Acta*, 1946, **29**, 1283.

‡ The term "capillary permeability" has been loosely used in medical literature, and is frequently confused with "capillary fragility," "capillary filtration," "extravasation," etc. The subject has been discussed recently by Landis.[10]

[10] Landis, E. M., *Annals N. Y. Acad. Sci.*, 1946, **46**, Art. 8, 713.

[11] Fukuda, T., *Arch. exp. Path. u. Pharmakol.*, 1932, **164**, 685.

[12] Czimmer, A. G., *ibid.*, 1936, **183**, 587.

[13] Jeney, A. V., Méhes, J., Sokoray, L., and Czimmer, A. G., *ibid.*, 1937, **187**, 553.

[14] Sokoray, L., and Czimmer, A. G., *ibid.*, 1938, **190**, 622.

[15] Muset, P. P., and Garcia-Valdecasas, F., *Estudiòs sobre la vitamina P. I. Acción antioxigeno de la vitamina "P"*, Consejo superior de investigaciones cientifícas, Madrid, 1944.

[16] Muset, P. P., *Introducción al estudio de la vitamina P*, 1 vol., 125 pp., Monografias Miguel Servet, Barcelona, 1945.

[17] Lavollay, J., *C. R. soc. biol.*, 1945, **139**, 270.

[18] Parrot, J., and Cotereau, H., *ibid.*, 1945, **139**, 1051.

[19] U. S. Department Commerce Rept. No. PB-981, p. 79, July, 1945.

moval of the isopropanol *in vacuo,* solution of the residual syrup in 15 cc of methanol and addition of this solution to 200 cc ether, gave a filterable solid. This was dried in the vacuum desiccator and ground in a mortar with several portions of fresh ether. The product was a yellow powder, soluble in $NaHCO_3$ solution.

The phthalates were synthesized by similar procedures, for example:

Hesperidin Acid Phthalate. Thirty-six grams of pure hesperidin were treated with 72 g phthalic anhydride in 150 cc dry pyridine, substantially as described above for rutin. The product was purified in much the same manner as in the above case, using butanol extraction and, after triturating the solid product with ether, was finally reprecipitated by acidification of its solution in $NaHCO_3$. The nearly white product was analyzed for phthalic acid residues and was found by 2 different methods to contain very close to 2 acid phthalate groups per molecule of hesperidin.

The sodium salts of the succinates and phthalates were prepared by neutralizing the acid salts with sodium bicarbonate, and were quite soluble and suitable for parenteral administration, in contrast with their parent compounds.

The esculin was purchased from the Mercantile Export and Import Co. of New York, and was a purified preparation. The hesperidin and its methylated chalcone were obtained from Mr. W. E. Baier of the California Fruit Growers' Exchange, Ontario, California. The hesperidin was purified by the formamide method of Pritchett and Merchant,[20] and the methylated chalcone was prepared by the method of Higby.[21] The *d*-catechin was a purified product prepared from gum gambir[§] by the method of Freudenberg,[22] and had a melting point 93.5°, which corresponds with the value described by Freudenberg[22] for the hydrated molecule. The epimerized *d*-catechin was supplied by Hoffmann-LaRoche, Inc., and was a brownish colored powder with an optical rotation of $[\alpha]^{25}_{D} + 23.2$ (C = 5, in 96% ethanol), equivalent to ca. 33% *d*-epicatechin. In addition, a brownish colored sample of epimerized *d*-catechin, "Catèchin actif" was obtained through the courteous cooperation of Prof. Jean Lavollay. Unfortunately, the sample was too small for an optical rotation determination. The *l*-epicatechin was a small sample obtained from Dr. Edwin F. Bryant of the California Fruit Growers' Exchange, Corona, California, who prepared it in Freudenberg's laboratory from *Acacia catechu* heartwood. "Catechin red" was prepared by the method of Etti,[23] and was rendered catechin-free by ether extraction. The adrenochrome was supplied by Hoffmann-LaRoche, Inc., and was prepared by the method of Buchnea,[24] by oxidation of epinephrine in methanol by means of silver oxide: $C_9H_9O_3N$, calc. C = 60.33, H = 5.06; found C = 59.03, H = 5.23, 5.22.

The capillary bed used was the rat mesoappendix preparation of Chambers and Zweifach.[25] The animals were anesthetized with 45 mg per kg of sodium pentobarbital intraperitoneally, and the bed exposed according to Zweifach's directions[26] and with the helpful suggestions of Dr. Chester Hyman of the Department of Aviation Medicine, University of Southern California. Inasmuch as it had been shown by Zweifach, Hershey, Rovenstine and Chambers[27] that the anesthetic had a deleterious effect on the capillaries, it was always given in a volume of not more than 0.1 cc. After 30 minutes, a booster dose of half the amount of the original dose was given to maintain the same depth of anesthesia. The preparation was bathed with Locke's adjusted to pH 7.6 with $NaHCO_3$, and containing 1% gelatin. The temperature of the solution, which dropped on the preparation at the rate of approximately one drop per second, was 37.5±0.2°C at the preparation. The animals used weighed from 120 to 244 g, with the majority weighing 150±20 g. The meso-appendix mesenteric membrane of heavier animals contained chains of fat which obscured the smaller vessels. All drugs were dissolved in Locke's solution. The *d*-catechin, epimerized *d*-catechins, as well as the adrenochrome were oxygen- and photo-sensitive. Decomposition of the catechin compounds was observed as a red-brown discoloration and the formation of a colored precipitate (catechin tannins). However this did not decrease the vasoconstrictor properties of these compounds within the periods of storage of the solutions investigated. The oxygen- and photo-sensitive solutions were freshly prepared for each experiment. All solutions were kept in the refrigerator in the dark when not in use. The solutions were placed on the membrane in doses of 0.1 cc, alternating between 0.05 γ of epinephrine (0.1 cc of $1{:}2 \times 10^6$) and the different dilutions of the compounds being tested, starting with the highest dilutions. The solutions were left on for 5 minutes, during which time the stream of the bathing fluid was interrupted. The animal preparation was considered "normal" only if a consistent and reversible vasoconstriction of the arterioles and meta-arterioles occurred with 0.05 γ of epinephrine, in accordance with one of the criteria established by Zweifach.[25–27] A total of 76 animals were employed.

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[20] Pritchett, D. E., and Merchant, H. F., *J. Am. Chem. Soc.,* 1946, **68**, 2108.

[21] Higby, R. H., *J. Am. Pharm. Assn.,* Sci. Ed., 1943, **32**, 74.

[22] Freudenberg, K., in G. Klein's *Handbuch der Pflanzenanalyse,* Bd.III, Spezielle Analyse, Teil, II, Organische Stoffe II, Julius Springer, Wien, 1932; see also Mason, F. A., *J. Soc. Chem. Ind.,* 1928, **47**, 269T.

[§] Supplied by the E. S. Miller Laboratories, Inc., Los Angeles.

[23] Etti, E., *Liebig's Annalen der Chemie,* 1877, **186**, 327.

[24] U. S. Dept. Commerce Rept. No. PB-47, 1946.

[25] Chambers, R., and Zweifach, B. W., *Am. J. Anat.,* 1944, **75**, 173.

[26] Zweifach, B. W., personal communication (directions for instructing new assistants).

[27] Zweifach, B. W., Hershey, S. G., Rovenstine, E. A., and Chambers, R., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 73.

Results and Conclusions. A saturated solution of rutin or hesperidin had no effect on the capillary bed. As these substances were very poorly soluble, all further work with them was done with the soluble succinates and phthalates. The other compounds investigated were relatively soluble. The results are summarized in Table I. The numbers signify the number of active vasoconstrictions observed over the total number of animals used. The heavy lines in Table I are drawn in the form of lateral histograms, to simplify comparison of relative activity of the various compounds.

The results indicate that some of the "vitamin P"-like substances were highly active vasoconstrictors when topically applied to the mammalian capillary bed, under the experimental conditions employed. This is in accord with the perfusion experiments of Fukuda,[11] Czimmer[12] and Jeney, Mehes, Sokoray and Czimmer,[13] but not with those of Sokoray and Czimmer[14] who reported vasodilatation by 1:5000 quercitrin in the isolated, perfused mesentery. If these workers had used the intact animal the results may have been different, but on the other hand intravascular contact by the compounds may differ from the topical effects described above. In agreement with Lavollay, Parrot *et al.*,[4–9] the catechins were the most active substances tested. According to these workers, *d*-epicatechin is the most active "vitamin P"-like compound, in decreasing capillary "permeability," capillary fragility, hemorrhagic diatheses and even shock symptoms. The present study indicates that epimerized *d*-catechins are no more active than pure *d*-catechin, and that a sample of *l*-epicatechin was as active or more so, at least in its direct effect upon the mesenteric capillary preparation investigated. Rutin, hesperidin, sodium rutin acid succinate, sodium hesperidin acid succinate, methylated hesperidin chalcone, esculin, and adrenochrome were inactive in the concentrations tested. Rutin and hesperidin sodium acid phthalates were slightly active, although negligibly so in comparison with the catechins.

The catechins were active even after partial decomposition. After decomposition had progressed, however, the catechins were damaging to the capillary bed at doses of 0.1 to 1.0 γ, since after they had been applied, the bed was unresponsive to epinephrine. Doses of 0.01 γ of the decomposing catechins were

TABLE I.
Effects of Topically Applied "Vitamin P"-like Substances on the Mammalian Capillary Bed.

Compound tested	No. of rats	Gamma added (in 0.1 cc)											Remarks
		100	10	1	1×10^{-1}	1×10^{-2}	1×10^{-3}	1×10^{-4}	1×10^{-5}	1×10^{-6}	1×10^{-7}	0	
		No. vasoconstrictions observed, over No. of rats used											
Na-rutin acid succinate	5	0/5	0/5	0/5	0/5	—	—	—	—	—	—	—	No effect in concns. studied
" phthalate	3	1/3	0/3	0/3	0/3	—	—	—	—	—	—	—	Constricts less than epinephrine
Na-hesperidin acid succinate	4	0/4	0/4	0/4	0/4	—	—	—	—	—	—	—	No effect in concns. studied
" " phthalate	4	3/4	1/3	0/4	0/4	—	—	—	—	—	—	—	Constricts less than epinephrine
Methylated hesperidin chalcone	4	0/4	0/4	0/4	0/4	—	—	—	—	—	—	—	No effect in concns. studied
Esculin	4	0/4	0/4	0/4	0/4	—	—	—	—	—	—	—	Same
Adrenochrome	3	0/3	0/3	0/3	0/3	—	—	—	—	—	—	—	Same
d-catechin	6	4/4	4/4	6/6	6/6	2/2	2/2	2/2	0/2	—	—	—	Sensitizes to subsequent doses
Epimerized *d*-catechin	6	2/4	2/4	2/4	6/6	6/6	0/2	—	—	—	—	0/2*	Same
"Catèchin actif"	5	3/3	3/3	5/5	5/5	5/5	2/2	2/2	0/2	—	—	0/2†	Same
l-epicatechin	3	—	3/3	3/3	3/3	3/3	2/2	3/3	1/1	1/1	1/1	—	Lowest dose not determined
Catechin red	2	0/2	0/2	0/2	0/2	—	—	—	—	—	—	—	Vasodilates, with stasis

* Distilled water.
† Ringer-Locke, with gelatin.

not damaging and the toxic effects of Lavollay's "Catèchin actif" were less noticeable than the sample of epimerized *d*-catechin obtained from Hoffmann-LaRoche. Catechin red was especially damaging in doses greater than 0.01 γ. It is probable that the damaging effect of the partially decomposed catechin preparations was due to the presence of catechin tannins formed during the decomposition. This is also supported by the observation by Parrot and Cotereau[18] that these colored substances, which they term "antivitamin P," antagonize the action of active catechin ("vitamin P"), and increase capillary fragility.‖

It is probable that some of the biological and clinical effects ascribed to "vitamin P" can be explained on the basis of a vasoconstrictor effect on the capillaries rather than to an indirect effect on these vessels through an epinephrine-sparing action[4-9] or by a direct action on capillary walls.[16]

Summary. 1. A mammalian capillary bed preparation was modified from the rat mesoappendix mesenteric preparation of Zweifach and Chambers, and used to estimate the relative activities of various topically applied "vitamin P"-like compounds on microscopically visible vasomotor responses of the vessels.

2. Rutin, rutin sodium acid succinate, hesperidin, hesperidin sodium acid succinate, methylated hesperidin chalcone, esculin and adrenochrome were inert in the concentrations used.

3. The sodium acid phthalates of rutin and hesperidin were slightly active vasoconstrictors.

4. The catechins were highly active vasoconstrictors, including *d*-catechins, epimerized *d*-catechins, and especially *l*-epicatechin, in doses as low as 0.0001 γ or less.

5. Catechin red (catechin tannin or anhydride) was highly toxic, inducing irreversible vasodilatation and stasis.

‖ Preliminary investigations in these laboratories indicate that catechin tannins, like tannic acid, are powerful endothelium poisons, producing marked hemorrhagic symptoms when administered intravenously, which supports the observations of Parrot and Cotereau.[18]

15911

Relative Gastro-Intestinal Stability of Carotene and Vitamin A and Protective Effect of Xanthophyll.*

W. C. Sherman. (Introduced by W. D. Salmon.)

From the Laboratory of Animal Nutrition, Alabama Polytechnic Institute, Auburn.

Previous work[1,2] from this laboratory has demonstrated that carotene administered to vitamin A-deficient rats is destroyed in the gastro-intestinal tract by the simultaneous feeding of unsaturated fat acid esters. The gastro-intestinal destruction of carotene was prevented by feeding α-tocopherol. Similar results have been reported by Quackenbush, Cox and Steenbock,[3] and by Hickman, Harris and Woodside.[4] Hove[5] has shown that the mucosa of the rat stomach contains an enzyme, carotene oxidase, which greatly accelerates the destruction of carotene *in vitro* in the presence of methyl linolate.

Under conditions used in biological assay procedures for vitamin A, the potency of vitamin A is reported to be about twice that of β-carotene on a weight basis. Values ranging from 0.3 μg[6] to 0.25 μg[7,8] of vitamin A have been reported to be equivalent to 0.6 μg of β-carotene (1 IU). However, no information is available on the relative potencies of vitamin A and carotene under conditions that would favor gastro-intestinal destruction of these factors.

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* Published with the approval of the Director of the Alabama Agricultural Experiment Station.

[1] Sherman, W. C., Proc. Soc. Exp. Biol. and Med., 1941, **47**, 199.

[2] Sherman, W. C., *Proc. Fed. Am. Soc. Exp. Biol.*, 1942, **1**, 134.

[3] Quackenbush, F. W., Cox, R. P., and Steenbock, H., *J. Biol. Chem.*, 1942, **145**, 169.

[4] Hickman, K. C. D., Harris, P. L., and Woodside, M. R., *Nature*, 1942, **150**, 91.

[5] Hove, E. L., *Science*, 1943, **98**, 433.

The possibility of xanthophyll exerting a sparing effect upon the gastro-intestinal destruction of vitamin A and carotene appeared worthy of investigation in view of the similarity in the molecular structure of xanthophyll and carotene and the susceptibility of both classes of compounds to oxidative destruction.

In the present investigation the response of vitamin A-deficient rats was used to determine the effect of xanthophyll upon the effectiveness of vitamin A alcohol, vitamin A acetate, and β-carotene under conditions that would favor gastro-intestinal destruction of these factors.

During the vitamin A depletion period, weanling rats were fed a diet of the following composition: alcohol-extracted casein 18, salt mixture No. 5[9] 4, sucrose 77, and cottonseed oil 1. The following amounts of vitamins were added in mg per kg of diet: calciferol 0.125, α-tocopherol 50, *i*-inositol 200, thiamin 2, pyridoxine 2, riboflavin 4, calcium pantothenate 10, niacin 20, and choline chloride 2000. Cottonseed oil and α-tocopherol were fed during the depletion period to minimize possible complications from the development of deficiencies of vitamin E and unsaturated fat acids. The rats were maintained upon the depletion diet until growth had ceased and ophthalmia had developed, at which time the daily feeding of supplements was started and the animals were changed to a test diet. The test diet differed from the depletion diet as follows: α-tocopherol and the cottonseed oil were omitted from the test diet and sucrose was increased to 78%. Fresh lard, which is known to contain only a small amount of antioxidants, was fed during the test period as a source of unsaturated fat acids.

The xanthophyll used in these studies was prepared from fresh Kentucky blue grass according to the method of Strain.[10] Strain's procedure was modified slightly in that the material, after being purified by adsorption from carbon disulphide on a chromatograph column of magnesium oxide, was crystallized twice from hot methanol without the addition of petroleum ether. The recrystallized xanthophyll was dried in vacuum over potassium hydroxide and calcium chloride. The adsorption spectrum of the xanthophyll in ethanol solution was determined by means of a Beckman spectrophotometer. The absorption curve had sharp maxima at 447 and 476 mμ and was qualitatively identical to the curve for lutein reported by Zscheile and associates.[11] The specific absorption coefficients of the xanthophyll at 447 and 476 mμ were 231 and 204, respectively. These and all other specific absorption coefficients throughout the spectrum were approximately 90% of the values found by Zscheile, indicating that the crystals were 90% pure lutein. It is probable that the crystalline material contained solvent, since it was not heated in drying. According to Zscheile,[11] lutein crystallized from carbon disulphide-ethanol retained approximately 10% of solvent, and heating at 84°C for 4 hours under high vacuum was necessary to remove the solvent.

Tests of the material by di-phasic fractionation between 90% methanol and petroleum ether showed that no carotene was present. When a *n*-hexane solution of the crystals was chromatographed through a column of alumina, there was no indication of carotene or

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[6] Mead, T. H., Underhill, S. W. F., and Coward, K. H., *Biochem. J.*, 1939, **33**, 589.

[7] Baxter, J. G., and Robeson, C. D., *J. Am. Chem. Soc.*, 1942, **64**, 2407.

[8] Baxter, J. G., and Robeson, C. D., *J. Am. Chem. Soc.*, 1942, **64**, 2411.

[9] Salmon, W. D., *J. Nutrition*, 1947, **33**, 155.

[10] Strain, H. H., Monograph, Carnegie Inst. of Wash., 1938.

[11] Zscheile, F. P., White, J. W., Jr., Beadle, B. W., and Roach, J. R., *Plant Physiol.*, 1942, **17**, 331.

TABLE I.
Responses of Vitamin A-Deficient Rats.

Daily supplements*	No. of rats	Avg wt gain at end of 3rd week g	 7th week g	Mortality %	Avg time to death,† days
2 μg carotene	14	22	33	14.3	74
2 ” ” + 5 μg xanthophyll	6	25	57	0	—
2 ” ” + 1 mg α-tocopherol	5	46	93	0	—
2 ” ” + 5 μg xanthophyll + 1 mg α-tocopherol	5	40	90	0	—
1 μg vit. A alcohol	19	—22	—50‡	100.0	25
1 ” ” ” ” + 5 μg xanthophyll	12	—17	—12	75.0	31
1 ” ” ” ” + 1 mg α-tocopherol	5	26	48	0	—
1.15 μg vit. A acetate	7	3	1	43.0	35
1.15 ” ” ” ” + 5 μg xanthophyll	7	5	29	14.3	37

* All rats received 0.2 ml of fresh lard with the above supplements.
† Only animals dying were included in the averages.
‡ This value represents the average weight gain at the end of the 5th week, beyond which no animal survived.

cryptoxanthin impurities. Nearly all of the pigment was contained in a single distinct band (lutein). A very small quantity of the pigment was strongly adsorbed to the upper surface of the alumina. This was probably a trace of other xanthophylls or oxidation products of lutein. The xanthophyll was dissolved in acetone for feeding in sufficient concentration so that the daily dose of 5 μg was contained in 0.2 ml of solution.

Crystalline vitamin A alcohol and vitamin A acetate (Distillation Products), pure β-carotene (prepared in this laboratory)[12] and α-tocopherol (Merck) were dissolved in a sufficient volume of *n*-hexane, so that the daily supplement of 1.0 μg of vitamin A alcohol, 1.15 μg of vitamin A acetate or 2.0 μg of carotene was continued in about 0.2 ml.

The supplements were fed each morning by pipetting the requisite amounts on a small amount of basal diet. After the solvents had completely evaporated, the fresh lard was added by pipette and the supplement jars were placed in the cages. The main portion of the basal diet was fed in the afternoon. Supplements were stored at 1°C. The carotene and vitamin A solutions were analyzed at weekly intervals in order to make any necessary changes in aliquots for feeding.

Results. The results of the feeding tests with rats are given in Table I. A marked superiority of 2 μg carotene over 1 μg vitamin A alcohol or 1.15 μg vitamin A acetate is evident under the conditions of this experiment. Although optimum growth was not obtained with 2 μg of carotene alone, the animals grew slowly and only 2 rats died during the 7-week test period. With 1 μg vitamin A alcohol all animals lost weight rapidly and died in an average of 25 days. Vitamin A acetate gave results intermediate between carotene and vitamin A alcohol; the mortality rate was 43% and the average time to death was 35 days.

Xanthophyll definitely improved the growth of rats receiving carotene or vitamin A acetate and reduced the mortality rate to 0. Xanthophyll added to vitamin A alcohol reduced the mortality rate and increased the average time to death. Rats receiving xanthophyll and vitamin A alcohol did not grow, but the loss in weight was considerably less and slower than was obtained with vitamin A alone. Although the growth stimulation obtained with xanthophyll was not as great as was obtained with α-tocopherol, only 5 μg of xanthophyll was fed as compared with 1 mg of α-tocopherol.

Discussion. The most logical explanation of the gastro-intestinal destruction of carotene and vitamin A in the absence of intestinal antioxidants is that the destruction is enzymatic in nature as is indicated by the results of Hove.[5] Nothing is known of the mechanism of the reaction or of the specificity of the enzyme, except that the presence of unsaturated fat acids is apparently required. The results reported in the present paper show that vitamin A alcohol is destroyed to a considerably greater extent than vitamin A acetate. The work of Hickman,[13] showing that vitamin A esters are more stable than free vitamin A when fish oils are aerated, is of interest in connection with these results. The effect of xanthophyll can probably be explained on the basis of the nonspecificity of the enzyme that results in the gastro-intestinal destruction of considerable quantities of the ingested xanthophyll, thereby sparing corresponding amounts of vitamin A or carotene. It is possible that, with certain human dietaries and animal feeds supplying vitamins A and E in suboptimum quantity, the protective effect of xanthophyll may be of practical significance.

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[12] Sherman, W. C., and Koehn, C. J., *Ind. and Eng. Chem.*, in press.

Summary. Vitamin A-deficient rats receiving a daily supplement of 1 μg of vitamin A alcohol with fresh lard in the absence of added α-tocopherol lost weight rapidly and 100% of the animals died. Carotene fed under the same conditions at a level of 2 μg/rat/day produced fair growth and a mortality rate of only 14.3%. Intermediate results were obtained with vitamin A acetate. The addition of xanthophyll apparently decreased the gastro-intestinal destruction of carotene, vitamin A alcohol, and vitamin A acetate and, thus, enhanced the response to the 3 vitamin A sources at the levels fed.

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[13] Hickman, K. C. D., *Ind. and Eng. Chem.*, 1937, **29**, 1107.

15912

Use of Dubos Medium for Culture of *M. tuberculosis* from Sputum.

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The liquid culture medium described by Dubos[1] can initiate rapid growth of *M. tuberculosis* from small inocula and maintain this growth in diffuse form. These media may offer, therefore, a great advantage for detection of *M. tuberculosis* in pathological material. Dubos tried his culture method on sputum (liquefied by sodium hydroxide), but concluded that "there is no indication that in its present form, the medium can be utilized with advantage for diagnostic work." At about the same time, Foley[2] reported successful culture of *M. tuberculosis* on Dubos medium from 165 pathological specimens (among them 31 sputa); thus demonstrating the suitability of the new medium for routine diagnosis. However, Foley emphasized the handicaps of some routine culture methods as applied in the use of Dubos media; "the digestion even for as short a period as 15 minutes with hydrochloric acid is liable to sterilize a specimen which contains only a few bacilli." "Little advantage results from the use of sulfadiazine to eliminate contaminating organisms in the specimens inoculated without digestion." It should be remembered that other authors, for instance Blacklock,[3] have studied the sterilizing effect of the alkaline digestion of sputa. Thus, the "digestion" (concentration) and the considerable elimination of contaminating bacteria without damage for acid-fast organisms, seem to be the essential condition for successful use of Dubos media in routine diagnosis.

Experimental. We have found in our preliminary experience with sputa mixed with a small number of *M. tuberculosis* organisms that the treatment of these sputa for 4 hours at 37° with a 2.5% solution of ammonium carbonate (1) does not sterilize even very small inocula (10^{-5} mg of an avian strain), (2) destroys many Gram-negative organisms, (3) liquefies the sputum readily (with few exceptions which require longer incubation and frequent shaking) and after centrifugation leaves a small volume of sediment, (4) promotes the rapid decomposition (with formation of ammonia) of ammonium carbonate and its neutralization by mucus thus rendering unnecessary its neutralization with acid which is required after digestion with sodium hydroxide.

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[1] Dubos, R. J., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 361; Dubos, R. J., *J. Exp. Med.*, 1946, **83**, 409.

[2] Foley, G. E., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 298.

[3] Blacklock, J. W. C., *Med. Res. Council Rep.*, 1932, p. 5.

To supplement by some other factor the relatively weak effect of ammonium carbonate on contaminating bacteria a solution of sodium penicillin in water was added to a concentration of 0.5 to 2 units per ml. Such amounts do not influence the growth of *M. tuberculosis* as was established by Abraham *et al.*[4] and confirmed in our comparative experiments on Dubos media with and without penicillin, inoculated with human or avian *M. tuberculosis* organisms, (laboratory strains). The addition of penicillin suppressed more or less completely the growth of contaminating organisms in cultures.

Methods. Cubes of ammonium carbonate ($NH_4HCO_3NH_2CO_2$) are used for preparation of 2.5% solution every 2 or 3 days. The solution is not stable.

The Dubos medium is prepared according to the formula: KH_2PO_4—1.0 g, $Na_2HPO_4 \cdot 12\ H_2O$—6.25 g; Na citrate—1.5 g, $Mg\ SO_4 \cdot 7\ H_2O$—0.6 g; Tween 80 5 ml of 10% solution; casein hydrolysate (N. Z Amine, type B, Sheffield) 20 ml of 10% solution; water to 1000 ml. The medium is adjusted to pH 6.8 and autoclaved for 15 minutes at 15 lb pressure. It becomes later slightly more alkaline by traces of ammonium carbonate in the inoculum.

Glassware. The medium is distributed in amounts of 5 ml in test tubes, 25 mm diameter. A 5% solution of bovine albumin (serum fraction V. Armour Laboratories) in 2% NaCl is sterilized by filtration, heated for 30 minutes at 56° and added in amounts of 0.2 or 0.3 ml to each tube.

The diluting fluid of Dubos consists of 2% glucose and 0.2% Vegex (we used up to 1% solution of Vegex) and is sterilized by autoclaving.

The penicillin solution is prepared by dissolving 100,000 units of sodium penicillin in 0.85% NaCl solution and preparing a 1:1000 dilution. Since the potency of penicillin in high dilutions decreases rapidly during storage, it is advisable to prepare frequently fresh dilutions from the stock solution.

Technic. 5 to 10 ml of the sputum were transferred from its container with some warm solution of ammonium carbonate (warmed to 60° in order to start production of ammonia) into a 50 ml centrifuge tube to which more solution was added so as to nearly fill the tube. The tightly plugged tubes were incubated at 37° for 4 hours, shaken every hour and centrifuged. The sediment was mixed with an equal volume of diluting fluid and inoculated in amounts of 1 to 3 ml into tubes containing each 5 ml of Dubos synthetic medium with 0.2 or 0.3 cc of albumin solution. Penicillin solution was added at the rate of 0.5 to 2 units per ml (the smaller amount was used when a solution was freshly prepared).

The inoculated tubes were incubated at 37°, smears from the cultures were prepared and examined in most series on the 8th and on the 15th day of incubation while in other series, on 10th to 12th days. Transplants of positive cultures to Loeffler's medium were made for eventual diagnosis of saprophytic bacteria in about half of the cultures. No saprophytic acid-fast bacilli were found.

Results. We have cultured 400 specimens of sputa from clinical patients sent for routine smear examination to the Diagnosis Laboratory of the New York City Department of Health. Only specimens containing an excess over that sufficient for the routine examinations were available for our experiments. Thus, the selection of the material was not based on any clinical criteria. We have obtained 116 positive cultures, 81 within 8 days and 35 within 9 to 15 days.

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[4] Abraham, E. P., Chain, E., Fletcher, C. M., Gardner, A. J., *et al.*, *Lancet*, 1940, **2**, 176.

(a) Microscopic appearance: The beginning of the growth manifested itself by the presence either of "nests" of 2 or 3 short acid-resistant slender bacilli bunched closely together or of very long slightly S-shaped, thick acid-resistant organisms. In the latter shape, the organisms formed large clusters and differed in appearance from the bacilli scattered diffusely all over the microscopic field.

(b) Contamination: If sufficient penicillin had been added to the medium the microscopic preparation presented a diffuse light blue background with a few contaminants scattered through the field; after transfer of such cultures to nutrient broth the contaminants grew sparingly or not at all. In several cultures, the staphylococci, streptococci and other cocci appeared intact, but in very small numbers and showed little or no growth after transfer of the cultures into broth. Some Gram-negative bacilli and molds were quite abundant in cultures but never obscured the presence of acid-fast organisms.

(c) Comparison of the results of sputum culture and routine microscopic examination of sputum concentrate. A large portion of each sputum used for comparison in these experiments had been concentrated in the routine laboratory treatment with an equal volume of tergitol and sodium hypoclorite and the sediment after centrifugation examined microscopically. We have compared the results of this method with our culture experiments. In no case was a sputum positive by microscopic examination (82 specimens) and negative in culture. On the other hand, somewhat more than 8% of all sputa were found negative on microscopic examination and positive by the culture method. Nearly all sputa positive on microscopic examination (70 out of 82 or 85.3%) yielded positive cultures within 8 days and only in 12 cultures (14.7%) were the organisms detected on further incubation. On the contrary of 34 sputa positive by culture alone only 11 (32.5%) produced detectable growth within 8 days and 23 (67.5%) within 10 to 15 days. These data suggest that the specimens positive on microscopic examination contain relatively large numbers of organisms and that this method is inefficient for sputa with small numbers of organisms which may be, however, detected by culture in Dubos medium. In our final experiments we have mixed known numbers (10^{-4} mg) recently isolated human strains with negative sputa and treated them by the above described method; in all cases positive cultures were obtained in Dubos medium with penicillin.

Summary. The use of a mild reagent (2.5% solution of ammonium carbonate) for homogenization and concentration of tuberculous sputa and the addition of penicillin (0.05 to 2 units per ml) to the culture medium permits the successful application of Dubos medium to the routine culture of *M. tuberculosis* from sputum. Of 400 examined sputa, 34 (8.5%) were positive by culture method and negative by microscopic examination. The advantage of the culture method is obvious. As compared with other culture methods, the use of Dubos media is inexpensive, easily learned by an average technician, suitable for daily examination of a large number of specimens, and what is more, offers the advantage of a rapid (within 8 to 15 days) culture diagnosis of sputa containing relatively small numbers of *M. tuberculosis*.

15913

Some Amino Acids of Chicken Erythrocytes.*

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The chicken erythrocyte is a cell which is readily available and ideal for various cytochemical and physiological studies. Furthermore, nuclei practically free of cytoplasm can be prepared in quantities sufficient for analysis by relatively simple procedures such as hemolyzing washed erythrocytes with saponin[1] or lysolecithin.[2] Morphological, chemical, and physiological studies on these erythrocytes and isolated nuclei from them allow for comparisons of nuclear and cytoplasmic functions which would be much more difficult to obtain on other types of cells. This report deals with some of the amino acids contained in the whole erythrocyte as determined by microbiological assays of hydrolyzates. Similar studies on nuclei are in progress.

Preparation of Erythrocytes. Blood samples were collected from healthy hens (White Rocks) by cutting the jugular vein and collecting the blood in an Erlenmeyer flask containing sodium citrate (5 mg per ml of blood) as an anticoagulant. The blood was transferred to 50 ml centrifuge tubes and centrifuged for 2 or 3 minutes at 3000 revolutions per minute. The supernatant plasma containing some erythrocytes and leucocytes was aspirated from the tubes. Ten ml of physiological saline (0.9% sodium chloride adjusted to pH 7.0 with M/15 phosphate buffer) was added to the tubes and the erythrocytes were resuspended. The tubes were centrifuged and the supernatant fluid and the layer of leucocytes which formed above the erythrocytes were removed by aspiration. Washing was repeated 5 additional times in order to free the erythrocytes of plasma proteins, as well as aid in removing the remaining leucocytes. After the last washing, a smear was prepared and stained with Wright's stain to check the absence of leucocytes in the preparation. The erythrocytes were transferred to evaporating dishes and dried over night at 105°C. The dried material was pulverized in an agate mortar and stored in weighing bottles. Five samples of dried erythrocytes, each weighing about 2.5 g, were prepared for this work.

Preparation of Hydrolyzates. One-gram samples of the dried erythrocytes were transferred to vials prepared by drawing out 150 x 22 mm Pyrex test tubes. To each vial 10 ml of 10% (by volume) hydrochloric acid was added. The vials were sealed and autoclaved for 10 hours at 15 lbs pressure. After cooling, the ampules were broken and the hydrolyzate washed into a beaker with a small amount of water. The hydrolyzate was neutralized with 5 N sodium hydroxide, the pH adjusted to 6.8, filtered, and diluted to a final volume of 50 ml with water. The hydrolyzates were stored under toluene in a refrigerator and, as a rule, were diluted with distilled water 1:25 ml or 1:50 ml before use.

Preparation of Amino Acid Standards.‖ Solutions of the *l* isomers of the various amino acids to be determined were prepared

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* This work was supported by funds from the Research Board, University of Illinois.

[1] Dounce, A. L., and Lan, T. H., *Science*, 1943, **97**, 584.

[2] Laskowski, M., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 354.

‖ Amino acids used as standards were furnished by Dr. Madelyn Womack, Department of Chemistry, University of Illinois.

† These organisms were obtained from the American Type Culture Collection, Georgetown University School of Medicine, Washington, D.C., where *Streptococcus faecalis* is listed as No. 9790, *Lactobacillus arabinosus* 17-5 as No. 8014, and *Lactobacillus delbruckii* LD5 as No. 9595. Folic acid used in the assay media was furnished by Dr. Beverly Guirard, University of Texas. The author is grateful for the suggestions of Dr. F. M. Clark, Department of Bacteriology, University of Illinois.

TABLE I.
Milligrams of Amino Acid per Gram of Dried Erythrocytes.

Sample	1	2	3	4	5	Average	%
Amino Acid							
Histidine	37.50	38.75	37.50	36.25	37.50	37.50	3.75
Arginine	42.50	42.50	45.00	39.00	48.60	43.52	4.35
Lysine	59.38	68.75	68.75	65.63	78.13	68.13	6.81
Leucine	87.50	85.00	92.50	92.50	102.50	92.00	9.20
Isoleucine	31.76	32.19	30.78	31.25	32.83	31.96	3.20
Valine	67.50	75.00	72.50	67.50	70.00	70.50	7.05
Methionine	11.25	13.75	11.88	13.13	12.50	12.50	1.25
Threonine	49.25	50.75	50.75	46.25	49.25	49.25	4.93
Tryptophane	12.80	12.60	13.00	13.00	12.80	12.84	1.28
Phenylalanine	41.25	42.50	45.00	41.25	42.50	42.50	4.25
Tyrosine	24.00	29.00	23.00	28.00	26.00	26.00	2.60

and stored under toluene in a refrigerator.

Bacteriological and Assay Procedures. Stab cultures[†] of *Streptococcus faecalis, Lactobacillus arabinosus* 17-5, and *Lactobacillus delbrückii* LD5 were maintained on the following medium: Bacto-tryptone 5 g, Bacto-yeast extract 3 g, dextrose 1 g, agar 15 g and water to 1 liter. Cultures were stored in a refrigerator and subcultured each month. Inoculum for each of the assays was produced by transferring a small amount of growth from a stab culture to a tube containing basal medium to which had been added the amino acid which was to be determined by the particular assay. This culture was incubated at 37.5°C for 24 hours, centrifuged, washed twice with 10 ml portions of sterile water, and suspended in 90 ml of sterile water. The tubes of the assay were inoculated with one drop of this bacterial suspension using a sterile hypodermic syringe as recommended by Black and Arnold.[3]

Medium for the determination of amino acids as developed by Stokes, Gunness, Dwyer, and Caswell[4] using *Streptococcus faecalis* was employed to determine histidine, arginine, lysine, leucine, isoleucine, and tryptophane. Valine and threonine were determined with *Lactobacillus arabinosus* 17-5 according to Hier, Graham, Freides, and Klein.[5] The medium of Schweigert, McIntire, Elvehjem and Strong[6] was used for methionine and phenylalanine and *Lactobacillus arabinosus* 17-5 was the assay organism. Tyrosine was determined according to the method of Gunness, Dwyer and Stokes[7] using *Lactobacillus delbrückii* LD5.

For the various assays used in this work 5 ml of basal medium, lacking the particular amino acid being determined, was placed in lipless tubes (180 x 22 mm) arranged in a metal rack. A standard series was prepared by adding various amounts, ranging from 0 to 5 ml, of the standard amino acid solution of the *l* isomer to tubes containing 5 ml of basal medium. To another series of tubes, each containing 5 ml of the basal medium, was added in duplicate 1.0, 2.0 and 3.0 ml of diluted hydrolyzate. The volume in each of the assay tubes was adjusted to 10 ml, if necessary, by adding water. All tubes were plugged and autoclaved 13 minutes at 15 lbs pressure. After inoculation, the tubes were incubated at 37.5° for 72 hours. Titration of the acid produced was carried out with 0.05 N sodium hydroxide with bromthymol-blue as an indicator. A curve was prepared from the titration data of each standard series by plotting the ml of 0.05 N sodium hydroxide against γ of the *l* isomer per tube. The concentration of each amino acid present in the hydrolyzate was estimated from these standard curves.

Results and Discussion. The results obtained from assays on 5 samples of dried chicken erythrocytes are presented in Table I. The values represent milligrams of amino acids per gram of dried erythrocytes.

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[3] Black, T. L., and Arnold, A., *Ind. Eng. Chem., Anal. Ed.*, 1940, **12**, 344.

[4] Stokes, J. L., Gunness, M., Dwyer, I. M., and Caswell, M. C., *J. Biol. Chem.*, 1945, **160**, 35.

[5] Hier, S. W., Graham, C. E., Freides, R., and Klein, D., *J. Biol. Chem.*, 1945, **161**, 705.

[6] Schweigert, B. S., McIntire, J. M., Elvehjem, C. A., and Strong, F. M., *J. Biol. Chem.*, 1944, **155**, 183.

[7] Gunness, M., Dwyer, I. M., and Stokes, J. L., *J. Biol. Chem.*, 1946, **163**, 159.

A survey of the literature indicates rather meager information on the amino acids contained in erythrocytes of various species of animals. However, analyses have been reported for several hemoglobins and stroma.[8] It is of interest to note the similarity in the results presented in Table I with those reported by Stokes, Gunness, Dwyer, and Caswell[4] for "blood meal." The chicken erythrocytes are somewhat higher in arginine and threonine and lower in histidine, lysine, leucine, and phenylalanine.

Summary. The following amino acids contained in the chicken erythrocyte have been determined by means of microbiological assays and are expressed as percentages of the dry weight: histidine 3.75, arginine 4.35, lysine 6.81, leucine 9.20, isoleucine 3.20, valine 7.05, methionine 1.25, threonine 4.93, tryptophane 1.28, phenylalanine 4.25, and tyrosine 2.60.

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[8] Block, R. J., and Bolling, D., *The Amino Acid Composition of Proteins and Foods,* 1945, Springfield.

15914

Renal Clearance of Essential Amino Acids: Threonine and Phenylalanine.

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Protein hydrolysates or amino acid mixtures are being employed widely today in clinical medicine. Present practices of administering these preparations either orally or parenterally in relatively large amounts have emphasized the need for additional information concerning the extent to which the various amino acids are reabsorbed by the kidney tubules at elevated plasma levels.

Previous publications from this laboratory in which microbiological methods of assay for the specific determination of amino acids in blood and urine were employed have been concerned with the renal clearances in dogs of the amino acids leucine, isoleucine, valine and tryptophane[1] and arginine, histidine, lysine and methionine.[2] This paper describes the renal clearances of threonine and phenylalanine, the remaining 2 amino acids that are essential dietary components for one or more mammalian species.

Methods. The physiological procedures employed in these experiments have been described in previous publications.[1,2] Creatinine clearances were used as a measure of glomerular filtration rate while clearances of para-aminohippuric acid determined at low plasma levels were employed as a measure of minimal renal plasma flow.

The experiments involving threonine and phenylalanine were designed to determine the clearances of the individual amino acids at normal postabsorptive blood levels followed by similar clearance periods at elevated plasma levels. Increased plasma levels were obtained by the use of priming doses and constant intravenous infusions of the compounds.

Microbiological determinations of the amino acids in plasma were carried out on protein-free filtrates prepared according to the method of Dunn *et al.*[3] Determinations

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[1] Beyer, K. H., Wright, L. D., Russo, H. F., Skeggs, H. R., and Patch, E. A., *Am. J. Physiol.,* 1946, **146**, 330.

[2] Wright, L. D., Russo, H. F., Skeggs, H. R., Patch, E. A., and Beyer, K. H., *Am. J. Physiol.,* 1947, **149**, 130.

TABLE I.
Renal Clearance Studies of *dl*-Threonine.

Dog 84, wt 17.0 kg.

				dl-Threonine				
Time hr:min	Renal plasma flow PAH cc/min	Glomerular filtration rate cc/min	Urine flow cc/min	Plasma conc. mg/cc	Amt filtered mg/min	Amt reabsorbed mg/min	Amt excreted mg/min	Clearance cc/min
Control: Post-absorptive but after priming dose of water.								
0:10	183	72.0	4.45	0.057	4.10	4.07	0.03	0.52
0:20	208	68.6	4.45	0.058	3.98	3.96	0.02	0.41
Priming 4.0 mg/kg—Maintenance 4.0 mg/kg/min—Infusion 3 cc/min.								
0:50		77.9	4.20	0.239	18.62	18.37	0.25	1.05
0:60	187	67.3	5.65	0.280	18.84	18.55	0.29	1.05
Priming 6.0 mg/kg—Maintenance 10.0 mg/kg/min—Infusion 3 cc/min.								
1:30	195	77.8	7.55	0.709	55.16	54.02	1.14	1.61
1:40		69.4	7.35	0.829	57.53	56.16	1.37	1.66
Priming 8.0 mg/kg—Maintenance 14.0 mg/kg/min—Infusion 3 cc/min.								
2:10		68.8	8.20	1.274	87.65	85.23	2.42	1.90
2:20	165	66.2	8.30	1.512	100.09	97.65	2.44	1.62

TABLE II.
Renal Clearance Studies of *dl*-Phenylalanine.

Dog 84, wt 17.3 kg.

				dl-Phenylalanine				
Time hr:min	Renal plasma flow PAH cc/min	Glomerular filtration rate cc/min	Urine flow cc/min	Plasma conc. mg/cc	Amt filtered mg/min	Amt reabsorbed mg/min	Amt excreted mg/min	Clearance cc/min
Control: Post-absorptive but after priming dose of water.								
0:20	209	70.3	1.10	0.037	2.60	2.58	0.02	0.56
0:30	198	73.5	3.10	0.042	3.09	3.07	0.02	0.55
Priming 6.0 mg/kg—Maintenance 8.0 mg/kg/min—Infusion 12 cc/min.								
0:60	150	69.9	4.80	0.349	24.39	22.82	1.57	4.50
1:10		76.1	4.95	0.436	33.18	30.90	2.28	5.24
1:20	160	81.4	5.45	0.439	35.73	33.29	2.44	5.56

on urine were carried out on suitable aliquots of the untreated material.

Threonine was determined microbiologically by the procedure of Stokes *et al.*[4] in which *Streptococcus fecalis* R was used as the assay organism. The extent of bacterial growth was determined turbidimetrically after an incubation period of 18-24 hours. Recovery of threonine from plasma was 105%.

The method of Stokes *et al.*[4] for the determination of phenylalanine was found satisfactory when applied to plasma but failed in the determination of phenylalanine in urine. There appears to occur in urine a material giving anomalous responses to phenylalanine with this medium such that quantitative recovery of the amino acid was not obtained. The method of Dunn *et al.*[5] employing a somewhat different basal medium was found satisfactory for the determination of phenylalanine in urine. A recovery of 100% was obtained.

It was necessary to use racemic amino acids in these experiments because the naturally-

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[3] Dunn, M. S., Schott, H. F., Frankl, W., and Rockland, L. B., *J. Biol. Chem.*, 1945, **157**, 387.

[4] Stokes, J. L., Gunness, M., Dwyer, I. M., and Caswell, M. C., *J. Biol. Chem.*, 1945, **160**, 35.

[5] Dunn, M. S., Shankman, S., and Camien, M. N., *J. Biol. Chem.*, 1945, **161**, 643.

occurring forms were not available to us. The results have been expressed in terms of the dl products used in the infusion solutions and used as standards in the assays. *Streptococcus fecalis* R, and *Lactobacillus casei* utilize only the naturally-occurring enantiomorphs of the amino acids studied.[4,5] Two dogs were used in these experiments.

Results and Discussion. The results have been summarized as protocols in Tables I and II.

Plasma levels of threonine were attained such that 100 mg of the amino acid were calculated as filtered per minute at the glomerulus. It was not practicable to administer larger amounts of phenylalanine than that employed because of the relative insolubility of the amino acid, limitations in the amount of fluid that could be continuously infused, and the nausea that accompanied the administration of the amino acid in large amounts.

At postabsorptive plasma levels of threonine and phenylalanine less than 1.0% of either amino acid that was filtered at the glomerulus appeared in the urine. When the plasma level of threonine was raised to over 10 times that of the postabsorptive state, more than 97% of the filtered amino acid was reabsorbed by the tubules, and clearances of only 1.0-2.0 cc per minute were obtained.

The capacity of the tubules to reabsorb phenylalanine was not exceeded by the maximal doses that could be administered. However, at the higher blood levels studied reabsorption of phenylalanine was only about 85% complete. It is possible that at plasma levels only slightly higher than were attainable in these studies the maximal rate of tubular reabsorption (Tm) might have been attained.

Previously reported clearance data concerning the amino acids commonly referred to as essential have indicated that they may be classified in 2 groups with respect to their reabsorption by the kidney tubules of the dog.[1,2] Leucine, isoleucine, valine, tryptophane, histidine, and methionine, when administered singly, were completely reabsorbed at all plasma levels practical for study while arginine and lysine were not well reabsorbed at elevated plasma levels. Tm values of 11 and 13 mg per minute respectively were obtained for arginine and lysine. The present studies would place threonine and phenylalanine in that group of amino acids that are well reabsorbed and for which no well defined threshold appears to exist.

Had the naturally-occurring forms of threonine and phenylalanine been available, it would have been desirable to use these forms in the studies reported in this paper. It would be anticipated that if an unnatural enantiomorph of an amino acid influences reabsorption of the natural form it should inhibit rather than enhance the process.

Summary. Renal clearance studies of threonine and phenylalanine in dogs have shown that the maximal rate of tubular reabsorption of these amino acids could not be exceeded by the administration of an amount of each amino acid sufficient to raise the plasma level to a value over 10 times that of the postabsorptive state.

15915

Some New Esters of Strophanthidin.

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During the last few years, 22 esters of strophanthidin were studied pharmacologically in this laboratory.[1-4] It was observed that more potent derivatives than strophanthidin could be prepared, and that 3-acetyl strophanthidin (identical with strophanthidin-3-acetate) was the most potent of the entire series. Additional investigations with several of these esters were carried out elsewhere.[5,6] To explore the changes of cardiac activity resulting from substitution in a single ester, 8 new derivatives of 3-acetyl strophanthidin were synthesized and tested intravenously in cats. They were 3-bromo-, 3-iodo-, 3-phenyl-, 3-phenoxy-, 3-methoxy-, 3-diethyl-, 3-trichloro-, and 3-trimethyl-, acetyl strophanthidin.

The strophanthidin used in the present investigation was obtained from the seeds of *Strophanthus kombé* according to the method of Jacobs and Heidelberger.[7] The esters were prepared by suspending the dry, powdered strophanthidin in a mixture of the desired acid chloride and equal parts of dry benzene and dioxane. The suspension was heated on the steam bath until complete solution had taken place and then poured into petroleum ether to terminate the reaction. In the preparation of the bromo- and iodoacetyl derivatives the benzene-dioxane solution was not heated above 55°C. Recrystallization of the products was carried out in ethanol solution. The results of the combustion analyses confirmed the purity of the compounds. Their melting points are recorded in Table I.

These new esters are sparingly soluble in water, and require ethanol for solution. In order to avoid the effects of alcohol, minimal amounts were employed for each of the compounds. It required 47.5% ethanol by volume to make a 1:1000 solution of 3-phenylacetyl strophanthidin; 76% for that of 3-iodoacetyl or 3-methoxyacetyl strophanthidin; and 95% for that of 3-bromoacetyl strophanthidin. Solutions of 1:500 were prepared with 3-diethylacetyl strophanthidin in 57% ethanol by volume; and with 3-trichloroacetyl and 3-trimethylacetyl strophanthidin in 95%. 3-Phenoxyacetyl strophanthidin was so insoluble that it was necessary to use absolute ethanol in order to make a 1:2000 solution.

Six of the above stock solutions were diluted with physiologic saline prior to administration to cats. The dilutions, governed by the toxicity and solubility of the strophanthidin esters, were as follows: 1:50,000 with the 3-diethylacetyl and 3-methoxyacetyl; 1:25,000 with the 3-trimethylacetyl, 3-iodoacetyl, and 3-phenylacetyl; and 1:20,000 with the 3-trichloroacetyl.

Etherized cats were injected with the diluted solutions by a continuous intravenous infusion until death occurred.[8] The rate of injection was 1 cc per minute with the 3-iodoacetyl, 3-phenylacetyl, 3-methoxyacetyl, and 3-trichloroacetyl strophanthidins; and 2 cc per minute with the 3-diethylacetyl and 3-tri-

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[1] Chen, K. K., and Elderfield, R. C., *J. Pharm. and Exp. Therap.*, 1942, **76**, 81.

[2] Steldt, F. A., Anderson, R. C., Maze, N., and Chen, K. K., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 198.

[3] Steldt, F. A., Anderson, R. C., and Chen, K. K., *J. Pharm. and Exp. Therap.*, 1944, **82**, 98.

[4] Chen, K. K., and Anderson, R. C., *J. Pharm. and Exp. Therap.*, in press.

[5] Gold, H., Otto, H. L., Modell, W., and Halpern, S. L., *J. Pharm. and Exp. Therap.*, 1946, **86**, 301.

[6] Lehman, R. A., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 372.

[7] Jacobs, W. A., and Heidelberger, M., *J. Biol. Chem.*, 1922, **54**, 253.

[8] Chen, K. K., Chen, A. L., and Anderson, R. C., *J. Am. Pharm. Assn.*, 1936, **25**, 579.

[9] Chen, K. K., Robbins, E. B., and Worth, H., *J. Am. Pharm. Assn.*, 1938, **27**, 189.

TABLE I.
Results in Cats.

Ester of Strophanthidin	Melting point, °C	No. of cats			Dose range to kill μg per kg	Mean (geometric) lethal dose ± standard error μg per kg
		Male	Female	Total		
3-Bromoacetyl	220-222	0	2	2	7651.0-7976.0	7803
3-Iodoacetyl	186-188	7	3	10	572.5- 762.7	677.8 ± 19.5
3-Trichloroacetyl	181-183	3	6	9	354.7- 663.4	453,9 ± 27.9
3-Trimethylacetyl	223-224	3	3	6	1422.0-2699.0	1799.0 ± 188.0
3-Methoxyacetyl	221-222	6	3	9	203.9- 521.6	334.5 ± 29.5
3-Diethylacetyl	207-209	6	4	10	607.2-1934.0	1047.0 ± 125.3
3-Phenylacetyl	204-205	6	4	10	558.1-1394.1	1061.0 ± 89.1
3-Phenoxyacetyl	231-232	5	1	6	235.0- 655.3	334.8 ± 48.7

methylacetyl strophanthidins. The 3-bromoacetyl and 3-phenoxyacetyl esters were administered in stock solutions owing to their insolubility in saline, the rate being 0.1 cc per minute for the former, and 0.05 cc per minute for the latter, both followed by 1 cc of saline through a 3-way stopcock after each injection. Of 62 cats the body weight ranged from 1.763 to 2.778 kg, averaging 2.184 kg. The number of animals used for 7 esters varied from 6 to 10 each. Only 2 cats were injected with 3-bromoacetyl strophanthidin since its activity was decidedly lower than the others.

All the 8 compounds retain their digitalis-like action as judged by bradycardia, arrhythmia, secondary tachycardia, and terminal ventricular tachycardia in cats during the slow intravenous injection of each. In Table I, the mean (geometric) lethal doses are shown in the last column. None of them is as potent as 3-acetyl strophanthidin which has a mean lethal dose of 186.6±24.6 μg per kg in cats.[1] By comparison of the toxicity values previously published[2] with those of the present series, it will be noted that the 3-chloroacetyl strophanthidins have the following order of activity: dichloroacetyl> trichloroacetyl > chloroacetyl. Considering strophanthidin-3-propionate and 3-*iso*butyrate as methyl- and dimethyl-, acetyl strophanthidins, respectively, it can then be seen that the 3 methylacetyl derivatives have the following order of activity: methylacetyl> dimethylacetyl>trimethylacetyl. In other words, chlorination of acetyl strophanthidin does not alter the cardiac activity in the same direction as methylation of the same. Of the 3 monohalogen substituents, the iodoacetyl derivative is the most active, and the bromoacetyl the least active. The high activity of 2 ether-like compounds, 3-methoxyacetyl and 3-phenoxyacetyl strophanthidins, is noteworthy although neither exceeds the activity of the parent aglycone, strophanthidin. Equally interesting is the low potency of 3-phenylacetyl as compared with 3-phenoxyacetyl strophanthidin, in the ratio of 1 to 3. A reverse order of activity, although not to the same extent, exists between 3-methylacetyl (identical with strophanthidin-3-propionate) and 3-methoxyacetyl strophanthidins.

Summary. Eight derivatives of 3-acetyl strophanthidin have been synthesized and studied pharmacologically in cats. None of the new compounds is as active as 3-acetyl strophanthidin, although 2 of them approach the parent aglycone, strophanthidin, in potency. The significance of certain structural changes against cardiac activity has been pointed out.

The authors are indebted to Misses Nila Maze and Marian H. Ellaby, and Mr. Harold M. Worth for their invaluable assistance in animal experiments.

15916 P

Lysozyme Content of the Stomach and Its Possible Relationship to Peptic Ulcer.*

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The bacteriolytic enzyme lysozyme has been shown to depolymerize and hydrolyze a mucopolysaccharide obtained from lysozyme-susceptible organisms.[1] The depolymerization of this substrate as measured viscosimetrically has been used as an accurate and speedy test for the assay of the enzyme.

Acetone desiccated mucosa of horse and hog stomachs were found by this method to contain 15.2 and 714 units of lysozyme per gram respectively (samples were obtained through the courtesy of Dr. R. H. Barnes of Sharp and Dohme, Philadelphia). Mucosa of the fundus, antrum, pylorus and duodenum of human stomachs resected for peptic ulcer were found to contain mean lysozyme titers of 12, 120, 316, and 213 units per gram of wet weight respectively. One case of ulcer of the duodenum showed the following lysozyme titers: fundus: 2, antrum: 200, pylorus: 224, and duodenum: 400 units per gram.

Assays of the gastric juice of 30 normal individuals and 29 unoperated ulcer cases showed mean lysozyme values of 7.69 and 14.3 units per cc respectively. The difference of the means is statistically significant. All cases with obstruction, as evidenced by intractable vomiting, had a mean lysozyme titer of 1.7 units per cc. This low value is probably explained by peptic destruction of lysozyme. Approximately 50 γ of crystalline pepsin inactivated (at 37°C) 81% of an equivalent amount of lysozyme in one hour and 92% in 2 hours. The mean lysozyme titers of the unoperated ulcer cases without the 7 obstructed individuals was 18.3 units per cc. This figure is statistically highly significant.

The mean lysozyme titers of the gastric juice on other groups of cases were: postgastrectomy (for ulcer), 7 cases—30.7; gastroduodenitis, 2 cases—23.8; marginal ulcers, 3 cases—33.1; postgastroenterostomy (for obstruction), 2 cases—9.1 units per cc.

It seems significant that in the 7 postgastrectomy cases no instance of hyperacidity was found, while 6 of the 7 individuals had a high lysozyme titer. The gastric juice of the 2 marginal ulcer cases which followed gastrectomy showed low or normal acid levels but high lysozyme titers (62.9-32.6 units per cc).

Because of the known beneficial effect of vagectomy on peptic ulcer, 5 cases were studied before and after operation. The mean fall in titer was 44.4%, ranging from 16.7 to 74.4%. This indicates that lysozyme production is, at least in part, influenced by nervous control.

Pure eggwhite lysozyme, in high concentrations, completely removed the surface mucus of the Pavlov pouch of an anesthetized dog when instilled over a period of 4 hours. The lysozyme was dissolved in pooled normal human gastric juice. A small ulcerated lesion resulted in the antral portion of this pouch, characterized by a destruction of the mucous cells within the gastric pits in the lamina propria. In another living dog, almost complete removal of the surface mucus was accomplished by instillation of eggwhite lysozyme dissolved in saline through a Levine tube into the functioning stomach. These observations were based on histological study of sections of the mucosa taken at the conclusion of the experiments.

Furthermore, a mucopolysaccharide frac-

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* This work was supported in part by the Josiah Macy, Jr., Foundation, New York.

[1] Meyer, K., and Hahnel, E., *J. B. C.*, 1946, **163**, 723.

tion was isolated from hog gastric mucosa which, on incubation with eggwhite or *ficus* lysozyme,[2] was hydrolyzed, yielding reducing sugars. The 2 known (neutral and acid) polysaccharides of gastric mucin[3] are not attacked by lysozyme.

Dodecyl sulfate, which has been reported to influence mucin production,[4] strongly inhibits eggwhite and gastric lysozyme. The latter, in extracts of human mucosa, is completely inactivated by M/1000 dodecyl sulfate.

The facts reported in this paper strongly suggest a role for lysozyme in the etiology of peptic ulcer.

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[2] Meyer, K., Hahnel, E., and Steinberg, A., *J. B. C.*, 1946, **163**, 733.

[3] Meyer, K., Smyth, E. M., and Palmer, J. W., *J. B. C.*, 1937, **119**, 73.

[4] Shay, H., Komarov, S. A., and Siplet, H., *Science*, 1947, **105**, 128.

15917 P

Lysozyme in Chronic Ulcerative Colitis.*

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It has been previously shown in experimental animals that there is a low lysozyme content of the colonic mucosa in contrast to that of the stomach and in assays of apparently normal segments of 3 human large intestines, surgically removed for carcinoma, the lysozyme concentration was similarly low (mean = 3.5 units/g tissue). However, following the observation that lysozyme was able to remove the surface mucus from the dog stomach,[1] the present investigation was undertaken to determine whether abnormal concentrations of this mucolytic enzyme were present in the feces of patients with chronic nonspecific ulcerative colitis.

Table I summarizes the results of the stool lysozyme determinations. It is to be noted that the concentration of the enzyme in the feces of the control individuals was low whether the determinations were made on specimens obtained following a normal bowel movement or after purging with magnesium sulfate or castor oil. Similarly, in 3 chronic ulcerative colitis patients whose disease necessitated ileostomy and colectomy, the lysozyme concentration of the ileal stools was uniformly low. Also, in the single patient with idiopathic diarrhea who failed to show any organic change in the mucosa of the gastro-intestinal tract, there was little lysozyme present in the stool. In marked contrast to the above noted findings was the elevated lysozyme concentrations in the fecal excretions of 12 patients in whom the diagnosis of nonspecific chronic ulcerative colitis was established by roentgen and proctoscopic examination and in whom exhaustive search for pathogenic bacteria and/or parasites was negative. Even more striking was the high titer of lysozyme found in specimens of mucus obtained from the rectosigmoid region of patients with this disease.

In some instances it was possible to collect 24-hour stool specimens so that the total lysozyme excreted per day could be determined. The highest total lysozyme content noted in the feces of the control group was 528 units in 24 hours, whereas lysozyme excretion in the colitis patients was as high

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* This work was supported in part by a grant from the Josiah Macy, Jr., Foundation, New York.

[1] Meyer, K., Prudden, J. F., Lehman, W. L., and Steinberg, A., Proc. Soc. Exp. Biol. and Med., 1947, **65**, 220.

TABLE I.
Lysozyme Assays of Normal and Pathological Stool Specimens.

Source	Individual lysozyme titers (units per g wet wt)			Mean lysozyme titer
Normal stools	0.5	9.4	4.4	2.7
	0.2	0.8	0.9	
Normal stools (after purging)	0.4	3.9	1.0	1.6
	0.8	1.3	0.1	
	1.6	3.8		
Chronic ulcerative colitis stools	28.3	48.7	22.3	56.0
	49.0	10.5	180.9	
	33.3	47.0	103.7	
	24.2	119.4	4.1	
"Mucus" from chronic ulcerative colitis patients	43.5	80.0	15.7	158.1
	167.0	466.0	176.5	
Ileal stools	0.1	2.0	3.6	2.8
Idiopathic diarrheal stool	5.0			

as 44,400 units in a comparable time period.

The experiments, although limited in number, indicate that lysozyme production is greatly increased in the diseased colon. The low titer of the ileal stools in the disease makes it unlikely that the enzyme is produced outside the colon. The high titer of the "mucus" points to the colonic mucosa as the source of the enzyme, although some microorganisms have been shown to produce small quantities of lysozyme.[2] We propose as a hypothesis that the pathogenesis of chronic ulcerative colitis may be due to a combination of local overproduction of lysozyme followed by a necrotizing action on the mucosa by the indigenous bacterial flora. We assume from analogy with the action of lysozyme on the gastric mucosa that the enzyme prepares the way for the necrotizing effect by a removal of the protective surface mucus. This hypothesis depends on the demonstration of a substrate of lysozyme in the colon.

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[2] Meyer, K., Palmer, J. W., Thompson, R., and Khorazo, D., *J. B. C.*, 1936, **113**, 479.

15918

Effect of Caronamide upon Penicillin Therapy of Experimental Pneumococcus and Typhoid Infections in Mice.

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It is now agreed that one of the major disadvantages of penicillin as a therapeutic agent is the rapidity with which the human and animal kidney excretes the drug from the plasma into the urine. Because of this rapid excretion, which occurs both by way of the renal tubules and the glomeruli,[1] relatively large doses must be given every few hours if a detectable plasma concentration is to be maintained. To obtain higher concentrations of penicillin in the plasma, very much greater quantities are required. The administration of both diodrast[2] and para-aminohippuric acid (PAH)[3,4] simultaneously with penicillin have been used to decrease the excretion of penicillin. Both of these substances, however, must be given in large amounts by continuous intravenous infusion.

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[1] Beyer, K. H., Peters, L., Woodward, R., and Verwey, W. F., *J. Pharm. and Exp. Therap.*, 1944, **82**, 310.

This communication reports experiments demonstrating the effect of a chemical compound that has been reported by Beyer[5] to offer a new approach to the problem of decreasing the urinary excretion of penicillin. The compound, 4′-carboxyphenylmethanesulfonanilide, will be reported by Sprague, Ziegler, Miller and Cragoe,[6] and has been called caronamide. It will be referred to by this name throughout this report. Unlike diodrast and PAH, which compete with penicillin on a mass-action basis for the penicillin transport mechanism of the renal tubular epithelium,[1] caronamide does not appear to be excreted by the tubules and is, therefore, believed to suppress penicillin excretion by blocking the specific enzyme system responsible for penicillin transport through the tubular cells. Because tubular elimination is apparently not a factor in the excretion of caronamide it remains in the blood for a longer period of time than does either diodrast or PAH. Therefore, it is possible to use intermittent administration of caronamide to suppress the excretion of penicillin.[7] For this purpose, the oral route of administration is effective since caronamide is rapidly absorbed from the gastro-intestinal tract. A more detailed discussion of the pharmacology, chemistry and mechanism of action of this drug will be published elsewhere.[5-9]

The pharmacological experiments of Beyer *et al.*, using dogs,[7] demonstrated that the intravenous or oral administration of caronamide could suppress and even eliminate the tubular excretion of penicillin when this antibiotic agent was given by either the intravenous or oral route. Although these experiments clearly indicated that greater therapeutic activity could be expected, the actual increase in the *in vivo* bacteriostatic effect produced by such enhanced and prolonged plasma penicillin concentrations obviously could not be determined by this type of experiment. It was, therefore, considered desirable to design experiments in which animals would be infected with lethal doses of microorganisms and treated with graded amounts of penicillin both alone and with caronamide. The survival rates in the groups of animals receiving various doses of penicillin with and without the drug were believed to offer a means of calculating the bacteriostatic dosage-equivalents of penicillin alone and penicillin with the drug. The ratio of these penicillin dosage-equivalents then could be used as an estimate of the *in vivo* bacteriostatic advantage derived from the suppression of penicillin excretion by the administration of caronamide.

Experimental. White Swiss mice weighing 16 to 22 g were used in all tests. An experiment was carried out using a strain of Type I pneumococcus as the infecting organism. Groups containing 20 mice were established, each mouse receiving, by intraperitoneal injection, 0.5 ml of a 1:3,000,000 dilution of a 6-hour broth culture. This amount of culture subsequently was shown, by titration in untreated control mice, to contain approximately 1000 minimum lethal doses of pneumococci. Animals which received combined treatment were given 10 mg of caronamide in 0.5 ml of a 0.5% gum tragacanth suspension by stomach tube and an intramuscular injection of 0.1 ml of 0.85% sodium chloride solution containing 2, 4 or 8 units of penicillin. The animals receiving only penicillin were given a similar intra-

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[2] Rammelkamp, C. H., and Bradley, S. E., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 30.

[3] Beyer, K. H., Flippin, H., Verwey, W. F., and Woodward, W., *J. Am. Med. Assn.*, 1944, **126**, 1007.

[4] Loewe, L., Rosenblatt, P., and Alture-Weber, E., *Am. Heart J.*, 1946, **32**, 327.

[5] Beyer, K. H., *Science*, 1947, **105**, 94.

[6] Sprague, J. M., Ziegler, C., Miller, C. S., and Cragoe, L. J., to be published.

[7] Beyer, K. H., Miller, A. K., Russo, H. F., Patch, E. A., and Verwey, W. F., *Am. J. Physiol.*, in press.

[8] Crosson, J. W., Boger, W. P., Shaw, C. C., and Miller, A. K., to be published.

[9] Beyer, K. H., Rapoport, M., Corneal, F. B., and Verwey, W. F., to be published.

TABLE I.
Survival Percentages of Mice Infected with Type I Pneumococci and Receiving Penicillin with Caronamide and Penicillin Alone.

Penicillin dosage,* units	Penicillin with caronamide†	Penicillin alone
2	0	—
4	35	—
8	84	—
16	—	5
32	—	10
64	—	65
128	—	85
PD_{50}	5.0 units	55 units

* Doses given intramuscularly every 6 hours for 9 doses.

† 10 mg given orally every 6 hours for 9 doses.

muscular injection containing 16, 32, 64 or 128 units of penicillin and an oral dose of 0.5 ml of the gum tragacanth suspending vehicle alone. All the animals, except the culture virulence controls, received this treatment immediately following infection and every 6 hours thereafter for 48 hours (9 doses). Treatment then was discontinued and the mice were observed for 5 additional days. The results of this experiment are given in Table I, which includes also the PD_{50} dose (dose protecting 50% of mice) calculated from the data by the method of Reed and Muench.[10] These latter figures represent the calculated amounts of penicillin that would have saved the lives of 50% of the mice treated by the 2 procedures employed in the experiment outlined above.

The data indicate that penicillin administered intramuscularly was approximately 11 times more effective in the prevention of death from pneumococcal infection when its excretion was decreased by the simultaneous oral administration of caronamide. Two other experiments similar to this, except that 10 mice were used in each group, have indicated equivalent dosage ratios of 6 and slightly greater than 16 in favor of the combined penicillin and caronamide therapy.

Inasmuch as pneumococci are known to be sensitive to very low concentrations of penicillin, experiments of the type described above were carried out using the "Panama 58" strain of *Eberthella typhosa*. Inocula of 10^7 organisms of this strain were found to be almost completely resistant *in vitro* to 2 units of penicillin, partially inhibited by 4 to 12 units and completely inhibited by 14 units per ml of culture medium. Mice were injected intraperitoneally with approximately 10^5 organisms, representing 1000 MLD's, in 0.5 ml of a 5% hog gastric mucin suspension. The groups of infected mice that received by mouth 10 mg of caronamide each were treated with intramuscular injections of 4, 20, 100 and 500 units of penicillin while those receiving no drug were given 20, 100, 500 and 2500 units of this antibiotic agent. As in the pneumococcal experiments, all mice were treated at the time of infection and every 6 hours thereafter for 48 hours. They were then observed for 5 days after the conclusion of the treatment period. The mice that were given penicillin alone received oral doses of gum tragacanth suspension containing no caronamide. Animals that were infected but untreated served as virulence controls.

The results of this experiment indicated that the PD_{50}'s of penicillin, when calculated from the survival percentages, were 320 units for mice receiving caronamide and 1370 units for mice not receiving this drug. The equivalent dosage ratio was, therefore, 4.2. This experiment was repeated using 2-fold increments between successive penicillin doses. The mice receiving 10 mg of caronamide

TABLE II.
Survival Percentages of Mice Infected with *E. typhosa* and Receiving Penicillin with Caronamide and Penicillin Alone.

Penicillin dosage* units	Penicillin with caronamide†	Penicillin alone
100	10.5	—
200	10	—
400	10	10.5
800	95	5.3
1600	—	30
3200	—	90
PD_{50}	515 units	1900 units

* Doses given intramuscularly every 6 hours for 9 doses.

† 10 mg given orally every 6 hours for 9 doses.

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[10] Reed, L. J., and Muench, H., *Am. J. Hygiene*, 1938, **27**, 493.

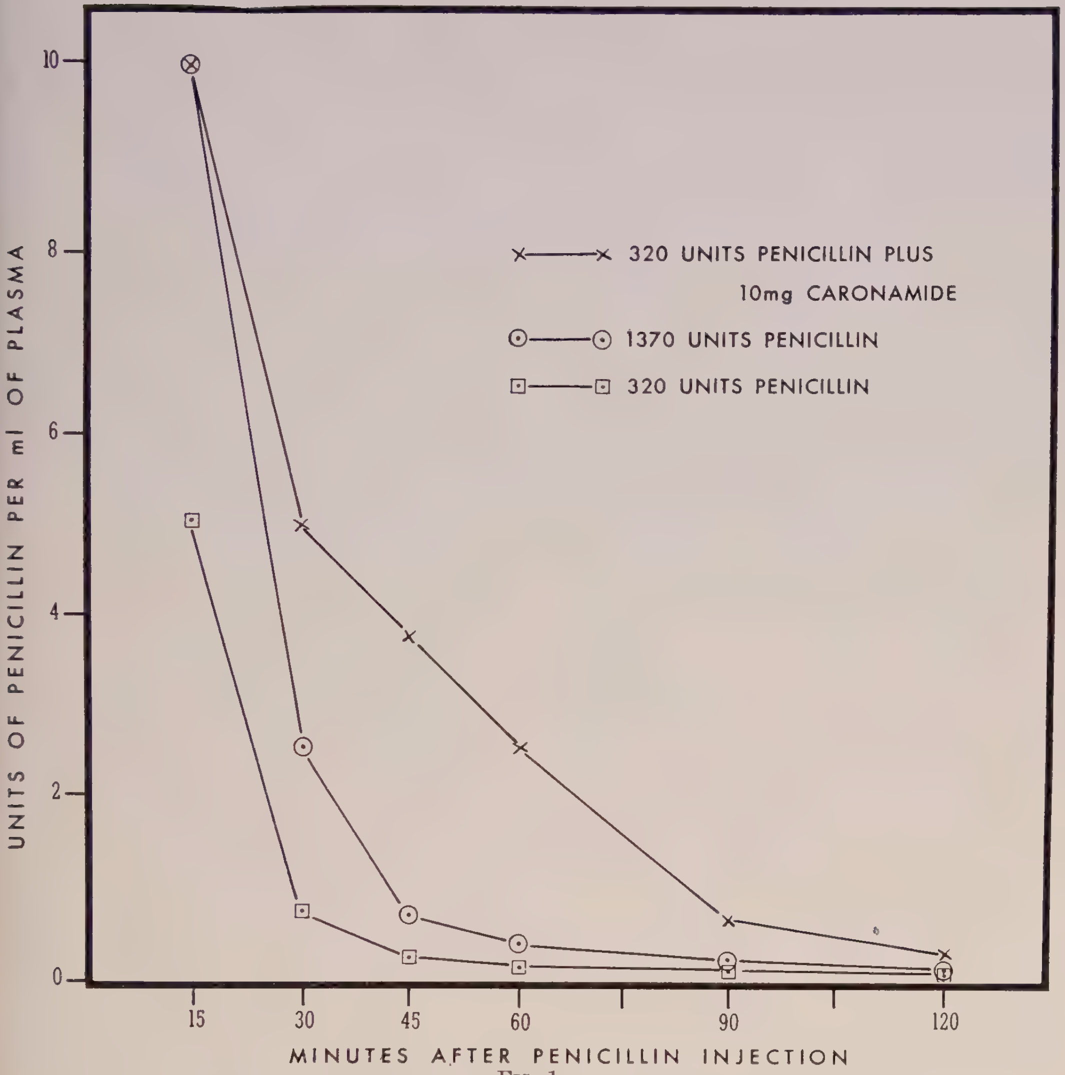

FIG. 1.

Plasma penicillin concentrations in mice following the administration of penicillin alone and penicillin with caronamide.

orally were given 100, 200, 400 and 800 units of penicillin and those receiving no drug were given 400, 800, 1600 and 3200 units. This change in the dosage increments was made for the purpose of increasing the accuracy of the calculations of the equivalent dosage. The results of this experiment are given in detail in Table II. The equivalent doses were 515 units when caronamide was given orally and 1900 without the drug. The ratio of equivalent doses was, therefore, 3.7.

Both the pneumococcus and the *E. typhosa* experiments demonstrated a very considerable increase in the effectiveness of penicillin with caronamide compared to penicillin alone. Experiments were then designed to determine whether doses of penicillin, with and without the drug, that were found to be equivalent in experimental therapy were also equivalent in respect to the height and duration of the plasma concentrations that they produced. Three groups of mice were established: One received 2 doses of 1370 units of penicillin intramuscularly with a 6-hour interval between doses, the second group received 2 doses of 320 units of penicillin intramuscularly according to the same schedule and 10 mg of caronamide orally, and the third group received similar doses of 320 units of penicillin alone. These amounts of penicillin were the equivalent doses calculated from the first experiment in which *E. typhosa* was used. The dosage of 320 units of penicillin without caronamide was included to determine the plasma concentrations produced by this amount of penicillin in the absence of drug. At intervals of 15, 30, 45, 60, 90 and 120 minutes after the second dose, 4, 5 or 6 mice in each group were exsanguinated under ether anesthesia within a 5-minute period and these individual citrated bleedings were pooled. Penicillin plasma concentrations were determined by a modification[1] of the method of Rammelkamp using a hemolytic streptococcus that permitted the detection of 0.019 unit of penicillin per ml of plasma. These data are summarized in the 3 curves of Fig. 1.

It will be seen that the administration of the therapeutically equivalent doses of penicillin with and without caronamide produced plasma penicillin concentrations of quite similar height and duration within the range to which this strain of *E. typhosa* was found to be sensitive in *in vitro* tests. However, as the plasma penicillin concentrations fell to lower levels, there was a marked decrease in the rate of disappearance of penicillin from the blood in animals receiving caronamide. This is illustrated in Table III where the periods of time are given during which the 3 dosage schemes maintained plasma penicillin concentrations at or above various levels. These data give an excellent illustration of the effect of caronamide on the height and duration of penicillin plasma concentrations.

TABLE III.
Duration of Plasma Penicillin Concentrations in Mice.*

Plasma penicillin concentration at or above	Dosage		
	Penicillin alone		Penicillin 320 units Caronamide 10 mg
	320 units	1370 units	
units	min	min	min
8	—	19	21
6	—	23	27
4	19	26	42
2	25	31	68
1	29	41	84
0.5	34	50	101
0.25	43	69	120

* Calculated from Fig. 1.

Discussion. The experiments that have been carried out clearly demonstrate that bacteriostatic action *in vivo* may be achieved with much smaller doses of penicillin when this treatment is combined with the oral administration of caronamide. In addition, it has been shown that the administration of caronamide causes penicillin to attain higher levels in mouse plasma and to remain there for a longer time than when a similar amount of penicillin is administered alone. Other work has indicated that caronamide produces this effect by causing the suppression of the tubular excretion of penicillin.[7] Since the drug has no demonstrable bacteriostatic action of its own and does not cause any enhancement of the *in vitro* bacteriostatic action of penicillin, it cannot be considered to act synergistically with this antibiotic agent. It is believed, therefore, that the increased effectiveness of intramuscularly-administered penicillin results from the influence of caronamide on the duration and magnitude of plasma and tissue concentrations of penicillin.

The strains of Type I pneumococcus and *E. typhosa* used in these experiments were selected as test organisms both because they produced satisfactory experimental infections in mice and because they were organisms having widely different sensitivities to penicillin. It is interesting to note that experiments with these 2 organisms resulted in different equivalent dosage ratios. These were 4 compared to 6-16 for the typhoid and pneumococcus experiments, respectively. The plasma penicillin concentration curves, when considered with the relative penicillin sensitivities of the 2 cultures, offer an explanation for this observation. The intensity of penicillin therapy can be tentatively represented as a function of the area under a curve where the concentration of penicillin is plotted against time and the baseline is the minimal penicillin concentration having any bacteriostatic effect upon the test organism. In the case of the *E. typhosa* strain, this baseline was approximately 2 units and with the pneumococcus it was 0.0008 unit. It can be seen that as the baseline is moved downward the area under the penicillin-caronamide curve increases disproportionally to that of the curve produced by the administration of penicillin alone. Thus it will be seen that the advantage produced by caronamide in penicillin therapy, though very considerable against organisms of relatively high resistance, becomes even greater against highly susceptible organisms.

Many bacterial infections that have been considered to be resistant to the usual clinical penicillin dosages have been found to be caused by bacteria sensitive to amounts of penicillin that, in these experiments, inhibited *E. typhosa.* Evans[11] has reported that, of 66 strains of *E. typhosa,* all but one were inhibited completely by 10-25 units of penicillin per ml of culture medium. While it is not possible to translate penicillin dosage in mice into human dosage, it seems reasonable to expect therapeutic effects in man at plasma penicillin concentrations similar to those found to be effective in mice. The studies carried out by Crosson, Boger, Shaw and Miller[8] have indicated that such plasma penicillin concentrations may be attained in man with moderate intramuscular doses of penicillin when caronamide is administered orally. It would, therefore, seem that the use of caronamide together with penicillin may offer a means of increasing the effectiveness of penicillin treatment and bringing more bacterial infections within the limits of practical penicillin therapy.

Summary. The oral administration of 4′-carboxyphenylmethane-sulfonanilide (caronamide) was found to enhance considerably the therapeutic effectiveness of intramuscularly-administered penicillin in mice experimentally infected with either Type I pneumococci or *E. typhosa.* This enhancement was 6- to 16-fold in the case of the pneumococcus experiments and approximately 4-fold for *E. typhosa.* These effects may be explained on the basis of the higher and more prolonged plasma penicillin concentrations when caronamide is administered, and do not indicate synergy between caronamide and penicillin. The significance of these observations in relation to penicillin therapy is discussed.

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[11] Evans, R. W., *Lancet,* 1946, **2**, 113.

15919 P

Low Sodium-Forced Fluid Management of Hypertensive Vascular Disease and Hypertensive Heart Disease.*

J. Marion Bryant and Elmira Blecha. (Introduced by Frank N. Wilson.)

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One hundred patients with essential hypertension were placed for periods of several weeks to 12 months on a diet of 2200 calories containing approximately 200 mg of sodium, 2.2 g potassium, 70 g protein, 80 to 175 g fat, 130 to 230 g carbohydrate, and vitamin supplements. A daily fluid intake of 3 liters was maintained. All were outpatients. Blood pressure readings were taken with the subject reclining, sitting and standing. They were made by one observer usually at the same time of day. No pretreatment blood pressure was below 170/100. Medical and dietary checks were ordinarily made once or twice a month.

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* Some of the observations upon which this paper is based were carried out with the aid of grants to Dr. Frank N. Wilson from the Horace H. Rackham School of Graduate Studies and the S. S. Kresge Foundation.

TABLE I.
Blood Pressure Changes in Hypertension with Surgical and Dietary Management.

	Smithwick's[2] post-operative classification.	Smithwick[2]*				Splanchnic[3]†				Low sodium‡			
Groups	Diastolic reduction	No. of cases	%			No. of cases	%			No. of cases	%		
I	30+	64	41.0	61.5	79.4	33	16.5	33	50	8	17.8	57.8	75.6
II	20 to 29	32	20.5			33	16.5			18	40.0		
III	10 to 19	28	17.9			34	17			8	17.8		
IV	to 9	17	10.9			50	25			5	11.1		
V	higher	15	9.7			50	25			6	13.3		

* Follow up on 156 patients with hypertension 1 to 5 years after surgery.

† Unpublished data. Study of 200 surviving patients of a total of 238 surgically treated with bilateral supradiaphragmatic splanchnicectomy and lower dorsal sympathetic ganglionectomy 1 to 2 years later.

‡ Forty-five unselected hypertensive patients treated for periods of several weeks to 14 months with 200 mg sodium diet—forced fluid regime.

There was a significant lowering of pressure (to or below 155/95) in approximately 20%, and of the diastolic pressure (to or below 95) in an additional 15% of the cases. A number of the patients who improved on this regime had failed to respond satisfactorily to a previous bilateral supradiaphragmatic splanchnicectomy and lower dorsal sympathetic ganglionectomy. The majority with symptoms typical of essential hypertension and hypertensive heart disease showed moderate improvement or were completely relieved of their discomfort. In most of these with angina pectoris the frequency and severity of the seizures were diminished. In all cases in which it was utilized orthodiography demonstrated a definite and progressive decrease in the size of the heart when it was enlarged. The inverted T waves frequently seen in hypertensive heart disease have in some cases become upright. Papilledema diminishes. Peripheral edema and pulmonary congestion invariably disappear. Relief of symptoms and the above described objective changes were not uniformly associated with a significant fall in blood pressure.

Several patients who originally displayed papilledema and moderately severe heart failure have been followed from 6 months to one year, have become symptom-free, and have shown a decrease in heart size and a definite fall in blood pressure. The blood pressure has not, however, reached the normal level.

Contrary to the experience of others[1] the older patients with long standing hypertension have experienced a more striking drop in blood pressure than younger individuals.

Digitalis has seldom been prescribed unless the patient was already digitalized at the time of the first visit. It is our impression that the response of patients with congestive cardiac failure on the basis of hypertensive heart disease placed on the regime described has often been more satisfactory than that of those treated with digitalis, mercurial diuretics and ordinary "low salt diets" containing 2 to 3 g of sodium chloride. Some patients who have been taking digitalis have improved when they discontinued this drug. This has happened especially in cases of angina pectoris. A number of patients not in apparent heart failure noted a definite decrease in nocturia in spite of a fluid intake of approximately 3 liters a day.

Few instances of symptoms suggestive of sodium privation have been observed and these have usually been controlled by reducing the fluid intake. One patient with very poor renal function developed orthostatic hypotension with bradycardia and azotemia. In one obese female a psychoneurotic crisis seemed to have been precipitated; it persisted after the diet was discontinued. This regime is tolerated in warm as well as cold seasons.

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[1] Grollman, A., *J. Am. Dietetic Assn.*, 1946, **22**, 864.

[2] Smithwick, R. H., *Arch. Surg.*, 1944, **49**, 189.

[3] Hettig, R. C., and Lyons, R. H., unpublished observations.

Preliminary studies suggest that the results obtained by treating essential hypertension with a diet low in sodium may prove to be better than those achieved by surgical procedures (Table I). This is true particularly in patients with far advanced heart and eye ground changes. The response seems to be similar in degree to that observed by Kempner[4] who uses drastic restriction of fluid (700 to 1000 cc), protein (20 g), fat (5 g), as well as sodium (200 mg), thus suggesting that his results, as previously pointed out,[5] may be primarily due to sodium restriction. Compared to that used by Grollmann *et al.*,[5] who obtained a significant reduction in blood pressure in 2 of 6 hypertensive patients, our diet appears less inconvenient, more palatable and contains about half as much sodium.

These observations confirm the early findings of Allen and Sherrill[6] in the treatment of arterial hypertension and the later observations of Schemm[7] in the management of heart failure. It should be emphasized, however, that failure may be expected unless the strictest cooperation and a complete understanding over a long period of time exists between patient, dietitian and physician.

It is suggested that rigid restriction of sodium seems to have a definite place at the present time, in the management of hypertensive vascular disease. These observations, in addition may have a more important bearing on the pathogenesis of this condition.

The authors wish to express their appreciation to Dr. L. H. Newburgh and Dr. Frank N. Wilson for their aid in carrying out this study.

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[4] Kempner, W., *Nor. Car. Med. J.*, 1945, **6**, 62.

[5] Grollman, A., Harrison, T. R., Mason, M. F., Baxter, J., Crampton, J., and Reichsman, F., *J. A. M. A.*, 1945, **129**, 533.

[6] Allen, F. M., and Sherrill, J. W., *J. Metab. Research*, 1922, **2**, 429.

[7] Schemm, F., *Ann. Int. Med.*, 1944, **21**, 937.

15920

Heterologous Transmission of a Human Lymphoma.

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Greene[1] was the first to transplant a human tumor into an animal by passage through the anterior chamber of the eye. Since 1939, at the suggestion of Dr. Lawrence Weld Smith, numerous attempts were made in this laboratory to transplant human tumors into animals by previous passage through tissue culture without any success. The work was resumed by the author and changes were made in the tissue culture medium and the type of mice used.

The tissue used for inoculation was obtained from a lymphnode of a patient whose diagnosis elsewhere had been "lymphosarcoma or aleukemic leukemia." The diagnosis on section here was "possible lymphosarcoma, leukemia cannot be excluded." Bone marrow examination did neither prove nor disprove the diagnosis of leukemia. The tumor was inoculated into a tissue culture medium consisting of the patient's own plasma, mouse embryo extract and Tyrode's solution at pH around 7.6. Growth was profuse and consisted mainly of lymphocytes, occasional blast forms and a few fibroblasts and reticulocytes. A small number of mitoses were noted.

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[1] Greene, H. S. N., and Murphy, E. D., *Cancer Research*, 1945, **5**, 269.

As mentioned above the patient's own plasma was used. In fact it had been observed in the study of tissue cultures in the past year, that certain tumors but more so those originating in the lymphnodes, grew more readily when the autogenous plasma was used instead of the habitual chicken plasma.* That there is a factor in the serum and plasma of cancerous patients peculiar to them has been noted by many research workers and some have used it to develop diagnostic tests.[2–4] Another change made in the preparation of the medium was the use of mouse embryo extract instead of calf embryo extract.

After 5 days the tissue culture was removed to a petri dish and cut into fragments measuring about 1 mm in size. Waste products (practically none), plasma clot and the tissue growth were included. Tyrode's solution was added so that each animal received .3 cc of suspension including 6 fragments of the tissue growth. No attempt was made to count the number of cells inoculated. Injection was done in the lower left quadrant part subcutaneously and part into the peritoneal cavity. The entire procedure was done under aseptic conditions. The animals used were a cancer-susceptible hybrid strain of female mice, 5 to 10 days postpartum. The control mice received a similar tissue culture medium without the cells.

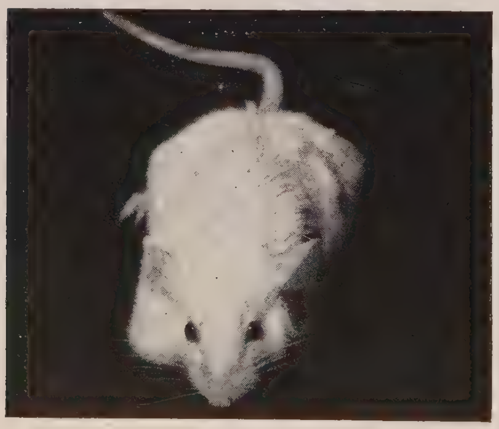

FIG. 1.
Mouse showing metastatic adenocarcinoma to the neck.

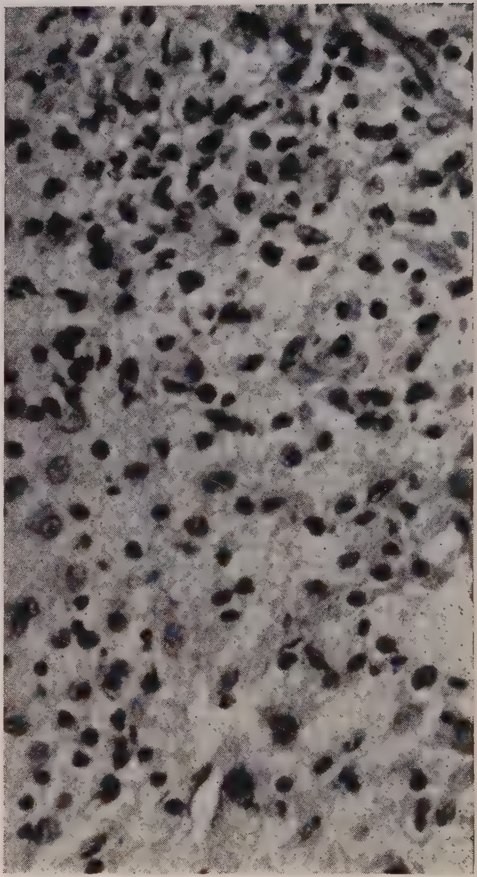

FIG. 2.
Primary tumor growth at the site of inoculation of mouse one (Fig. 1). × 200.

Three weeks later one of 4 mice developed a nodule at the site of inoculation. Six weeks later the tumor measured one cm in diameter and was firm and adherent to the skin. At this time a second tumor developed in the neck on the opposite side of the primary tumor (Fig. 1). The mouse was operated on under ether anesthesia and the left lower quadrant tumor removed. It was moderately firm, hemorrhagic and adherent to the skin and underlying tissues.

On section the upper surface of the tumor nearest to the region of inoculation, was seen

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* Paper in preparation.

[2] Mendeleeff, P. *Bull. d. l'Ass. Fr., p. l'Et. du Cancer*, 1938, **26**, 1.

[3] Carratala, A. T., *Bol. Inst. de med. exp. para el estud. y trat. d. Cancer*, 1944, **21**, 183.

[4] Roskin, G., *Am. Rev. Sov. Med.*, 1946, **4**, 111.

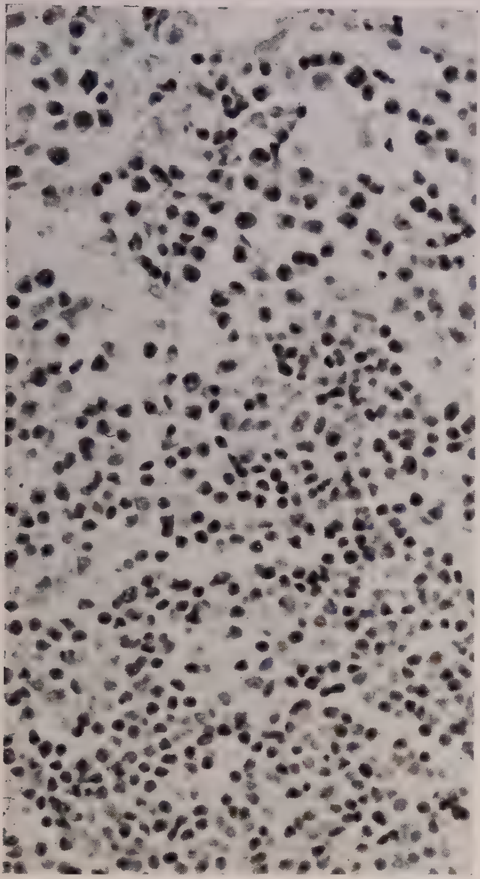

FIG. 3.
Human lymphoma, diagnosis questionable. × 200.

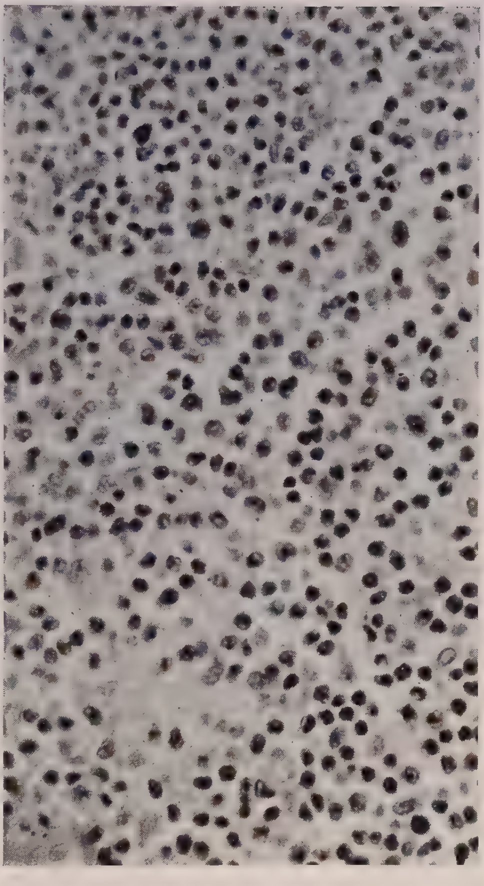

FIG. 4.
Primary tumor growth at the site of inoculation in mouse two. Note similarity with human tumor. × 200.

to consist of a fine network of reticulum-like stroma infiltrated with lymphoid cells, similar to those observed in the human tumor (Fig. 2 and 3). Adjacent to it and forming part of it was breast tissue. The lining epithelial of the acini had undergone malignant alteration so that the greatest mass of the tumor was formed by an adenocarcinoma in which little stromal element was present. Atypical mitoses were encountered.

A week later the neck tumor was operated on and this was likewise adenocarcinoma such as was found in the first tumor. Another tumor with rapid development now appeared on the back on the same side as the neck tumor. It was freely movable. The mouse was beginning to lose weight and was found dead 9 weeks after inoculation. The third tumor was likewise a carcinoma but much less differentiated than the previous ones. There was practically no stroma but sometimes a few round cells were noted in between the tumor cells.

The second mouse developed a nodule at the site of inoculation 6 weeks after the injection. It was yellowish grey in color and had a delicate vascular pedicule arising from the parietal peritoneum. The liver was markedly enlarged and was studded with delicate salmon pink nonelevated areas. On section the tumor nodule was similar in appearance to the human tumor presenting the same difficulty in diagnosis (Fig. 4). The liver was infiltrated throughout by the tumor cells (Fig. 5).

The third mouse developed a tumor about 6 weeks after inoculation but this was more a thickening of the subcutaneous tissue. The

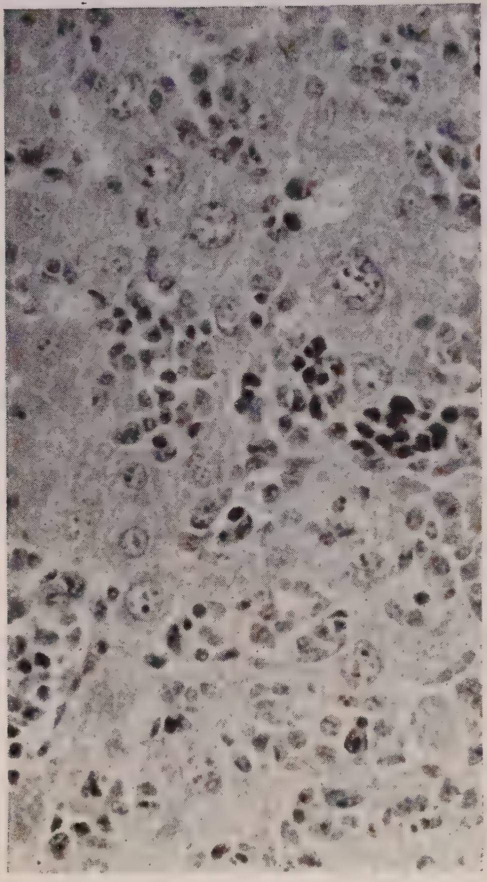

FIG. 5.
Metastasis to the liver of tumor shown in Fig. 4. × 200.

mouse was killed. The liver was slightly enlarged but grossly showed no evidence of tumor. On section the subcutaneous thickening was found to be actually formed by squamous pearls and normal breast tissue. Some of the cells were distorted and there was a marked hyperkeratosis. The picture suggested precancerous alterations.

The fourth mouse died 3 days after inoculation of intestinal obstruction due to mechanical perforation at the time of inoculation. One control mouse died 7 weeks later of pneumonia. The other 2 remained well.

Transmission of the carcinoma developed in the first mouse to other mice has been repeated thrice without much difficulty. Transmission of the lymphoma of the second mouse has not been entirely successful though not without interest. Direct transmission was unsuccessful. It was found expedient to pass this tumor back to tissue culture. Of the 8 animals then inoculated with the tissue culture 3 have shown disturbances in the white blood cell count. Of these 2 show a normal white count but the differential count reveals 86% lymphocytes none of which were abnormal. One mouse showed a white count of 32.000 with no apparent change in the differential count. Control blood counts were done and the variations always present taken into account. On all tumors, tissue cultures and other procedures bacterial studies were made. They were always negative.

Discussion. The diagnosis of the lymphoma has been and still is a difficult problem to the pathologist. Its transmission to animals has remained unsuccessful. In the above experiments it has been shown that under certain specified conditions it may be possible to transplant such a tumor and induce in the adjacent tissues a tumor of a different nature. It is true that the growth of the transplant was reduced yet sufficient to elicit tumor growth at the site of inoculation. Simultaneous growth of an undetermined lymphoma and an epithelial tumor at the same site had never been observed by the author in man. Recently Dr. Simpson brought to me a slide for examination in which such a tumor co-existed at the same site with a squamous cell carcinoma the latter metastasizing independently to the liver. I believe the growth and induction of these tumors under the above mentioned conditions is due to a concomitant action. This will require further verification.

Summary. Under specified conditions a human unclassified lymphoma was transmitted to mice by previous passage through tissue culture.† An unclassified lymphoma was produced in the mouse and an adenocarcinoma was induced in the adjacent breast tissue demonstrating the lability of these tumors.

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† Since this paper has been written another very similar case has occurred.

15921

Susceptibility of Convalescent Ferrets to Reinfection with Influenza Virus in Absence of Specific Antibodies.*

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It has been suggested[1] that during the early stages of convalescence from influenza virus infection, resistance to reinfection might be related more closely to a non-specific cellular refractoriness than to immunological activity of the blood. That suggestion is supported by the demonstration[2,3] that, following the intranasal inoculation of ferrets with the PR8 strain of influenza virus, the nasal epithelium was destroyed and was replaced by one of abnormal type, and that 7 to 8 days after the infection the abnormal epithelium was resistant to injury by the reinoculation of the same strain of influenza virus, and, also, was resistant to injury by a chemical stimulus which completely destroyed the nasal epithelium of the normal ferret. However, under the conditions of those experiments, animals which possess resistant nasal epithelium in all probability also would possess antibodies specific for the inoculated virus. It has not been shown that those animals would be more resistant to infection than would a normal animal of the same species, if inoculated with a strain of virus against which they possessed no antibodies.

In the experiment to be reported ferrets were infected with either influenza A or influenza B virus. Then, they were tested for immunity by intranasal inoculation of those viruses during that period of convalescence when resistant nasal epithelium would be expected to be present.

Pools of allantoic fluid infected either with the Czech strain of influenza B virus or with the PR8 strain of influenza A virus were collected and stored in the CO_2-ice chest until immediately before use. Twelve ferrets were anesthetized by intraperitoneal injections of nembutal. While anesthetized, they were bled from the heart and then were given 1.5 cc of inoculum intranasally. Six of the animals were infected with the Czech (B) strain of virus and 6 were infected with the PR8 (A) strain. One week later, 3 of the Czech infected animals and 3 of the PR8 infected animals were tested for immunity by intranasal inoculations with the Czech virus; the other 6 animals were tested by intranasal inoculations with PR8 virus.

In addition to the bleedings made at the start of the experiment, blood was obtained immediately preceding and one week following the second inoculation. The virus neutralizing antibody content of each serum was determined by means of the mouse protection test; the titers are expressed in terms of the initial dilution of serum which protected 50% of the mice from death[4]. The data are shown in Chart 1 and in Table I.

Chart 1 shows that the ferrets were susceptible to reinfection with the heterologous virus when inoculated 7 days after the initial infection. For example, all of the animals convalescing from infection with Czech, showed a definite febrile response when challenged with PR8. Also, the animals originally infected with PR8 responded, when subsequently inoculated with Czech, with a temperature course that was little different from that shown by normal animals inoculated with that virus. On the other hand, none of the animals gave a febrile reaction when reinoculated with the homologous virus.

Table I shows that by the time of the

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* This investigation was aided by a grant from the John and Mary R. Markle Foundation.

[1] Francis, T., Jr., *The Harvey Lectures*, 1941-42, The Science Press, Lancaster, Penn., 1942, p. 69.

[2] Francis, T., Jr., and Stuart-Harris, C. H., *J. Exp. Med.*, 1938, **68**, 789.

[3] Stuart-Harris, C. H., and Francis, T., Jr., *J. Exp. Med.*, 1938, **68**, 803.

[4] Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

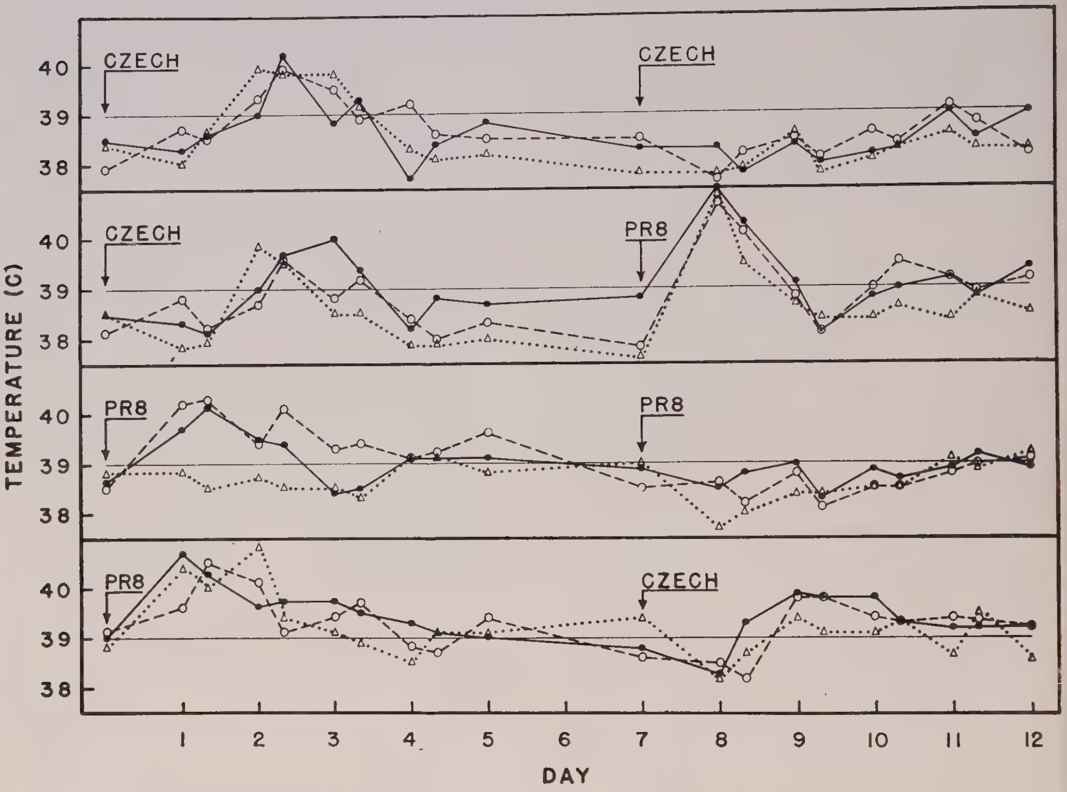

CHART 1.
Temperature courses following intranasal inoculation of influenza virus.

TABLE I.
Antibody Titers and Febrile reactions Following Intranasal Inoculation of Influenza Virus.

	1st day				8th day				15th day	
Ferret No.	Antibody titers Czech	Antibody titers PR8	Virus strain used for inoc.	2nd to 7th day. Fever	Antibody titers Czech	Antibody titers PR8	Virus strain used for inoc.	9th to 14th day. Fever	Antibody titers Czech	Antibody titers PR8
1	0	0	Czech	+	90	0	Czech	—	1000+	0
2	0	0	”	+	47	0	”	—	1000+	0
3	0	0	”	+	47	0	”	—	1000+	0
4	0	0	”	+	74	0	PR8	+	1000+	305
5	0	0	”	+	54	0	”	+	1000+	360
6	0	0	”	+	22	0	”	+	1000+	360
7	0	0	PR8	+	0	98	”	—	0	1000+
8	0	0	”	+	0	178	”	—	0	1000+
9	0	0	”	—	0	360	”	—	0	1000+
10	0	0	”	+	0	305	Czech	±	23	1000+
11	0	0	”	+	0	360	”	+	74	1000+
12	0	0	”	+	0	218	”	+	19	1000+

second inoculation, all of the ferrets had developed antibodies specific for the virus used for the original infection, while none of the animals possessed demonstrable antibodies reactive with the heterologous virus. Thus, during the period of convalescence, when a resistant nasal epithelium would be expected to be present, the animals were susceptible to reinfection with a strain of influenza virus against which they did not possess antibodies, although they were immune to clinical reinfection with a strain of influenza virus against which they did possess antibodies. If the mechanisms of infection with those 2 viruses are the same, non-specific cellular resistance resulting from infection with one virus should have conferred increased resistance to infection with the antigenically distinct virus. The results, therefore indicate that following an original influenza virus infection either (1) non-specific cellular refractoriness plays little, if any part in the resistance of ferrets to reinfection with that virus or (2) the mechanisms of infection with the A and B viruses are not the same.

It is of interest that when PR8 was used for inoculation (Chart 1), the animals previously infected with Czech showed a higher fever, an earlier peak and a less prolonged febrile reaction than did the normal animals. If that difference in response was not due to chance these results might be interpreted as an indication that the convalescing animals were even more susceptible to infection than were the normal ferrets.

Summary. Ferrets which had been infected with either influenza A or influenza B virus were tested for immunity to those 2 viruses on the eighth day of convalescence. They were susceptible to infection when reinoculated with the heterologous virus against which they possessed no antibodies, but were immune to infection when reinoculated with the homologous virus against which they possessed antibodies. If the mechanisms of infection with the A and B viruses are the same the results indicate that non-specific cellular refractoriness plays little part in the resistance of ferrets to reinfection with influenza virus.

15922

Observations on the Action of Streptomycin *in vitro* (I).*

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Since the discovery of streptomycin by Waksman,[1] a number of reports on the sensitivity of microorganisms to this antibacterial agent have appeared.[2-8] The sensitivities have been expressed in terms of units per cc or micrograms of streptomycin base per cc necessary for inhibition of growth. The sensitivities reported have varied widely depending upon the individual strain tested. Strains which have been considered sensitive to streptomycin and which have been inhibited in some laboratories by a few tenths of a microgram per cc have in other laboratories required from 1 to 10 μg per cc or more for inhibition. These differences have depended upon the experimental conditions, and upon the criteria of inhibition used.

It was early demonstrated by Waksman,[2,3] Donovick,[9] and others[10,11] that medium influences the sensitivity of an organism to streptomycin. Age and density of culture were recognized as significantly affecting sensitivity.[7,11] The significance of these factors has been further emphasized by the recent work of Berkman and his coworkers.[12]

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* A part of this work was presented before the Society of American Bacteriologists, May, 1946.

[1] Schatz, A., Bugie, E., and Waksman, S. A., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 66.

[2] Waksman, S. A., Reilly, H. C., and Schatz, A., *Proc. Nat. Acad. Sci.*, 1945, **31**, 157.

[3] Waksman, S. A., and Schatz, A., *J. Am. Pharm. Assn.*, Sci. Ed., 1945, **34**, 273.

[4] Buggs, C. W., Bronstein, B., Hirschfield, J. W., and Pilling, M. A., *J. Am. Med. Assn.*, 1946, **130**, 64.

[5] Helmholz, H. F., *Proc. Staff Meeting, Mayo Clinic*, 1945, **20**, 357.

[6] Feldman, W. H., and Hinshaw, H. C., *Am. J. Path.*, 1946, **22**, 640.

[7] Alexander, H. E., *J. Pediat.*, 1946, **29**, 192.

[8] Keefer, C. H., *J. Am. Med. Assn.*, 1946, **132**, 4, 70.

[9] Donovick, R., and Rake, G., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 224.

[10] Bondi, A., Dietz, C. C., and Spaulding, E. H., *J. Bact.*, 1946, **52**, 150.

[11] Hobby, G. L., Lenert, T. F., and Hyman, B., *J. Bact.*, 1946, **51**, 606.

Despite recognition of the fact that sensitivity could be altered by such factors as these, no standardized procedure for determination of the sensitivity of organisms to this agent has been utilized.

The present study was undertaken in an attempt to determine the range of sensitivity of various bacterial species to streptomycin, using a standardized procedure, and to determine certain of the factors which may influence the apparent sensitivity of an organism to streptomycin.

Method. Except when otherwise specified, a beef infusion medium[†] buffered at pH 7.8 was used throughout. This medium contained a relatively high concentration of phosphates which may decrease to some extent the activity of streptomycin. However it was felt advisable to use this in view of the fact that it is sufficiently rich to support the growth of practically all pathogenic organisms.

Six-hour plain broth cultures were used except when otherwise indicated. These cultures were prepared from freshly cultivated 16-18 hour broth cultures, and were diluted with broth to a constant density immediately prior to use. A density equivalent to a MacFarland $BaSO_4$ No. 1 standard and allowing 70 to 78% transmission on a Photovolt Lumetron No. 400 was arbitrarily chosen as standard. This density corresponds to a concentration of 2-400 million organisms per cc for the majority of strains tested. The sensitivity of a standard strain of *E. coli* was determined simultaneously with each series of unknown organisms.

For each organism tested, a series of 12 tubes were set up containing 0.01 to 0.1 cc, 0.15 cc, and 0.2 cc of a broth solution containing the equivalent of 100 μg of streptomycin[‡] per cc. The total volume of each tube was adjusted to 0.5 cc with sterile broth and 0.5 cc of a 10^{-3} dilution of the standardized culture was then added to each. The final concentration of organisms was therefore about 150,000 per cc; the final concentration of streptomycin varied from 1.0 to 20 μg per cc. In the case of highly sensitive organisms, a solution of streptomycin containing 25 μg per cc was at times necessary whereas for more resistant organisms, a solution containing 1000 to 2000 μg per cc was used. Unless otherwise specified, impure commercial streptomycin sulfate was used. Incubation was carried out at 37°C. for a period of 72 hours. The amount of growth was recorded at 24, 48, and 72 hours. The sensitivity of an organism was accepted as the least amount of streptomycin causing complete inhibition of growth, as evidenced by absence of gross turbidity, after 72 hours' incubation. It was recognized however that this did not necessarily indicate a bactericidal level.

Experimental. Using this procedure experiments were carried out (1) to demonstrate the effect of variation in culture on the sensitivity of an organism to streptomycin, and (2) to demonstrate the effect of medium on the sensitivity of an organism. In addition the sensitivity of 84 freshly isolated strains belonging to 10 different species was tested against impure streptomycin sulfate. Certain of these strains were also tested for compar-

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[12] Berkman, S., Henry, R. J., and Housewright, R. D., *J. Bact.*, 1947, in press.

[†] This medium was first used by Dawson[13] for cultivation of pneumococcus variants. It has since been widely used in a number of laboratories for various purposes including the primary isolation of a wide variety of pathogens. It readily supports growth of many organisms that are often difficult to cultivate in the usual beef infusion mediums. Lean chopped beef (1 lb per liter of water) is allowed to infuse at 5°C for 18 to 24 hours. The mixture is then boiled for 15 minutes, and filtered through cotton cloth. Sodium phosphate (Na_2HPO_4; 4 g/liter) and neopeptone (10 g/liter) are then added, the mixture boiled for 15 minutes, and filtered through filter paper while hot. After adding sufficient water to bring the volume to 1 liter, the pH is adjusted to 8.0 with 2N NaOH and the mixture boiled long enough to clear (no longer). The medium is then tubed and autoclaved at 15 lb pressure for 20 minutes.

[‡] All streptomycin used throughout this study was prepared by Chas. Pfizer and Co.

TABLE I.
Bacteriostatic Action of Streptomycin on a Standard Strain of *E. coli in Vitro.*

Streptomycin preparation	Potency µg/mg	Sensitivity in µg/cc* 24 hr	48 hr	72 hr
I	249	4.5	6	6
		7	7	7
II	418	7	8	8
		6	8	8
		7	7	8
		4	4	4
		5	5	5
		5	5	5
		4	4	4
III	802	5	5	5
		5	5	5
		9	9	9

* Sensitivity = Least amount of streptomycin causing complete inhibition of growth.

TABLE II.
Bacteriostatic Action of Various Preparations of Streptomycin on a Standard Strain of *E. coli in Vitro.*

Streptomycin preparation	Potency µg/mg	Sensitivity in µg/cc* 24 hr	48 hr	72 hr
I	249	5.8	6.5	6.5
II	455	6.5	7.8	7.8
III	404	7.9	7.9	7.9
IV	452	4.0	5.0	5.0
V	418	5.8	6.1	6.2
VI	802	6.3	6.3	6.3

* Sensitivity = Least amount of streptomycin causing complete inhibition of growth.
Values given represent average of 2 to 9 determinations made on separate days.

ison against the crystalline $CaCl_2$ double salt of streptomycin, and against highly purified streptomycin sulfate prepared from a crystalline salt.

Sensitivity of a Single Strain of E. Coli. The sensitivity of a given strain of *E. coli*§ was determined, by the method previously described, against three preparations of streptomycin on a number of days. As indicated in Table I, the sensitivity varied from 6.0 to 7.0 µg per cc against preparation I, from 4.0 to 8.0 µg per cc against preparation II, and from 4.0 to 9.0 µg per cc against preparation III.

Sensitivity of a Single Strain of E. Coli to 6 Preparations of Streptomycin Sulfate. In subsequent experiments the sensitivity of a single strain of *E. coli* was tested against six different preparations of streptomycin sulfate varying in potency from 249 to 802 µg per mg.

The sensitivity after 24 hours' incubation at 37°C. varied from 4.0 to 7.9 µg per cc. The action of streptomycin was only bacteriostatic, the amount necessary for inhibition increasing on prolonged incubation.

The sensitivity did not vary greatly with different preparations of streptomycin sulfate. A preparation containing 802 µg per mg and prepared directly from a crystalline salt of streptomycin was no more effective against this strain of *E. coli* than were more crude preparations. The variation in sensitivity to different preparations was no greater than the variation in sensitivity to a single preparation on different days. (Table II).

Effect of Concentration of Organisms on Sensitivity to Streptomycin. In view of the fact that the sensitivity of the same strain cultivated in the same medium was not absolutely constant from day to day, experiments were carried out to determine the effect of the density of the culture on its sensitivity to streptomycin.

The density of both 6 hour and 16 hour cultures of 2 strains belonging to different species—a standard strain of *E. coli* and a freshly isolated strain of *K. pneumoniae*—were diluted to the equivalent of a $BaSO_4$ No. 1 standard (78% transmission Photovolt Lumetron No. 400). This concentration was equivalent to 200-400 million organisms per cc. Six hour cultures were also diluted to the equivalent of a $BaSO_4$ No. 4 standard (50% transmission) whereas the 16 hour cultures were diluted to correspond to the density of a $BaSO_4$ No. 6 standard (28% transmission). The sensitivity of each to streptomycin was determined using 10^{-1} to 10^{-8} dilutions.

More constant and more complete inhibition of growth was obtained using 6 hour cultures. The number of µg per cc necessary to cause complete inhibition of growth decreased with a decrease in the number of organisms per cc. Using dilutions of 10^{-5} or greater (equivalent to less than 15,000 organisms per cc of final test solution), differences in the number of organisms had little effect on the

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§ We are indebted to Dr. Selman Waksman for the strain of *E. coli* used in these experiments.

TABLE III.
Effect of Concentration of Organisms on Sensitivity to Streptomycin *in Vitro.*

Strain	Culture age, hr	Density, $BaSO_4$	Sensitivity in µg/cc* Dilution of culture 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
E. coli (W.)	16	No. 1	10	5	5	4	2	2	2	2
		No. 6	10	10	5	4	2	2	2	2
	6	No. 1	10	10	5	2	2	2	2	2
		No. 4	10	10	10	5	2	2	2	2
K. pneum. (Lund.)	16	No. 1	10	10	5	2	2	2	2	1
		No. 6	10	10	5	4	3	2	2	2
	6	No. 1	10	10	5	3	2	2	2	2
		No. 4	10	10	10	4	2	2	2	2

* Sensitivity expressed in terms of least amount of streptomycin (µg/cc) necessary to cause inhibition of growth.

amount of streptomycin necessary for inhibition. In lower dilutions, a 10-fold difference in concentration of organisms was often sufficient to cause a 2-fold difference in the sensitivity of the organism when tested in the same medium under the same experimental conditions. (Table III).

The concentration of organisms, and to a less extent the age of the culture, is critical. Indeed it is more critical than in the case of penicillin—a 10,000-fold change in the concentration of an organism generally being essential to cause a 2- to 4-fold difference in its sensitivity to penicillin.

Variation in Sensitivity of Organisms Within Individual Strains. Throughout these studies, occasional irregularities resulted from the appearance of resistant cells at irregular intervals in the test series. In view of the fact that there is no evidence as yet that these cells differ in their pathogenicity, they cannot be ignored. However, the difference in sensitivity due to the appearance of such cells is no greater, in most cases, than the difference in sensitivity of a single strain when tested on different days or when tested against different preparations of streptomycin. (Table IV).

TABLE IV.
Influence of Resistant Cells on Sensitivity of Whole Culture to Streptomycin *in Vitro.*

Strain	Sensitivity in µg/cc (72 hr) Susceptible cells*	Resistant cells†
E. coli (W.)	4.5	7
” ” (Hopk.)	5.0	9
” *typhosa* (McM.)	7.0	9
” ” (Ur.)	3.5	9
K. pneumoniae (Yer.)	4.5	7
Strep. viridans (Tor.)	2.5	10
Staph. aureus (K.C.)	5.0	10
” ” (K.S.C.)	5.0	8
” ” (Hand.)	10.0	200

* Least amount of streptomycin necessary to inhibit growth of apparently sensitive cells.

† Least amount of streptomycin necessary to inhibit growth of entire culture.

Effect of Peptone on Sensitivity of E. Coli to Streptomycin. A comparison of the sensitivity of the standard strain of *E. coli* was made in (1) a freshly prepared beef infusion broth, (2) Bacto-nutrient broth containing yeast extract, and (3) McLeod's synthetic medium for growth of *E. coli.* The sensitivity of this strain was greatest in the Bacto-nutrient broth which contained only a low concentration of beef extract, peptone, and yeast extract at a pH of 6.9. In all 3 mediums, however, growth was inhibited by 5 µg of streptomycin per cc.

Using freshly prepared beef infusion broth, McLeod's synthetic broth, and a modified McLeod's medium in which the asparagin was replaced by neopeptone in amounts comparable to those present in the infusion broth, the sensitivity of varying dilutions of the standard strain of *E. coli* to streptomycin was tested.

The concentration of organisms ranged from a 10^{-1} to 10^{-8} dilution of a standard culture. Growth was inhibited in dilutions of 10^{-2} to 10^{-8} in beef infusion broth, and in dilutions from 10^{-3} to 10^{-8} in McLeod's medium containing asparagin. No inhibition

TABLE V.

Effect of Neopeptone on Action of Streptomycin (*E. coli*).

Basal medium	Amt of peptone (%)	Amt of streptomycin (μg/cc)	Amt of growth 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
McLeod's	0	0	++++	++++	++++	++++	++++	++++	++++	++++
,,	0	5	++++	++++	++++	—	—	—	—	—
,, *	0.2	0	++++	++++	++++	++++	++++	++++	++++	++++
,, *	0.2	5	++++	++++	++++	++++	++++	++++	++++	++++
Beef inf.	1.0	0	++++	++++	++++	++++	++++	++++	++++	++++
,, ,,	1.0	5	++++	++++	—	—	—	—	—	—

* McLeod's synthetic medium containing no asparagin. Neopeptone used in place of asparagin.

of growth occurred in McLeod's medium containing 0.2% neopeptone in place of asparagin. (Table V). Similar results were obtained using 1.0% neopeptone in McLeod's medium.

The inhibition of streptomycin action by peptone was in striking contrast to the effect obtained in beef infusion broth containing an equal concentration of the same preparation of peptone and containing the same concentration of glucose. That peptone may alter the oxidation-reduction potential of a medium was shown by Dubos.[14] It has been demonstrated by Geiger, Green and Waksman[15] and by others[9,12,16] that such alteration in the oxidation-reduction potential of the medium may inhibit the action of streptomycin. It is conceivable that the inhibitory effect of peptone on the antibacterial action of streptomycin may be due at least in part to such an alteration in the oxidation-reduction potential of the medium. Beef infusion broth apparently contains however one or more substances which will counteract the effect of the peptone on the action of streptomycin.

An inhibitory effect similar to that observed with peptone was also obtained when asparagin was replaced by methionine, cystine HCl, and tyrosine. No inhibition of streptomycin action occurred with the concentrations of leucine, glutamic acid, tryptophane, glucose, or paraminobenzoic acid tested. Certain other of the amino acids were tested but were in themselves inadequate to support growth of the organism in the synthetic medium used.

It is apparent that the medium used in testing the sensitivity of an organism to streptomycin is extremely important. The fact that certain growth stimulating substances such as peptone may have an inhibitory effect on streptomycin seems highly significant. Since large differences in sensitiv-

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[13] Dawson, M. H., *J. Path. and Bact.*, 1935, **39**, 323.

[14] Dubos, R., *J. Exp. Med.*, 1930, **52**, 331.

[15] Geiger, W. B., Green, S. R., and Waksman, S. A., *J. Bact.*, 1946, **51**, 634.

[16] Denkelwater, R., Cook, M. A., and Tischler, M., *Science*, 1946, **102**, 12.

ity levels may result from the use of different mediums in different laboratories, it is essential that the medium is indicated or that a reference standard is used. In a constant medium the variations in sensitivity of a given organism are not significantly great from day to day provided age and density of culture are controlled.

Sensitivity of Freshly Isolated Strains. In subsequent experiments the sensitivity of 84 freshly isolated strains was tested in beef infusion broth using a 10^{-3} dilution of a 6 hour culture diluted to 78% transmission as previously described. The sensitivity was accepted as the least amount of streptomycin necessary to cause inhibition of growth as evidenced by absence of turbidity after 72 hours at 37°C. The sensitivity was not expressed in terms of the actual number of μg per cc necessary for inhibition of growth, but in terms of the ratio of the number of micrograms necessary to inhibit the unknown organism being tested to the number necessary to inhibit the standard strain of *E. coli* in the same medium on the same day. Expressing sensitivity in terms of such a ratio does not eliminate differences in the culture from day to day or differences due to the appearance of occasional resistant cells, but it will eliminate differences caused by medium. The sensitivity of the standard strain of *E. coli* under such conditions is then 1.0 at all times.

TABLE VI.
Comparison of Bacteriostatic Action of Streptomycin on Various Species of Bacteria *in Vitro.*

Species	No. of strains tested	Avg sensitivity* 24 hr	Avg sensitivity* 72 hr
H. influenzae	1	0.2	0.2
B. mycoides	1	0.4	0.4
K. pneumoniae	5	0.3	0.5
Aer. aerogenes	1	0.6	0.6
E. typhosa	7	2.6	5.5
E. coli	10	1.6	2.2
Streptococcus	4	2.3	3.4
Salmonella	5	2.3	3.4
Staph. aureus	44	1.0	3.5
Pseud. pyocyaneus	6	13.9	24.1

* Sensitivity = avg of ratios of least amount of streptomycin (μg per cc) causing complete inhibition of growth of unknown strain to least amount (μg per cc) causing inhibition of a standard *E. coli* strain on the same day.

TABLE VII.
Comparison of Sensitivity of Various Strains Within a Species to Streptomycin *in Vitro.*

Species	Strain No.	Sensitivity (72 hr)*
E. coli	1	0.2
	2	0.6
	3	0.7
	4	0.7
	5	0.8
	6	1.0
	7	1.0
	8	5.6
	9	7.5
Ps. pyocyaneus	1	5.6
	2	7.5
	3	100.0

* Sensitivity = ratio of least amount of streptomycin (μg per cc) causing complete inhibition of growth of unknown strain to least amount (μg per cc) causing inhibition of standard *E. coli* strain on same day.

TABLE VIII.
Difference in Bacteriostatic Action of Streptomycin on Individual Organisms Within a Strain of *Staph. aureus.*

Strain	No. of colonies tested	Sensitivity* 24 hr	Sensitivity* 48 hr	Sensitivity* 72 hr
K.L.	2	0.3	0.3	0.5
		0.2	4.3	18.8
K.N.	2	0.6	0.6	0.8
		4.2	5.0	22.5

* Sensitivity = ratio of least amount of streptomycin (μg per cc) causing complete inhibition of growth of unknown strain to least amount (μg per cc) causing inhibition of standard *E. coli* strain on same day.

The average sensitivity of 10 strains of *E. coli,* other than the standard strain, was 2.2 μg per cc. These strains were 2.2 times as resistant as the standard strain. The strains of *H. influenzae, B. mycoides, K. pneumoniae,* and *Aer. aerogenes* tested were more sensitive whereas the streptococci (all of which were enterococci with the exception of one strain), staphylococci, Salmonella, and Pseudomonas strains tested were more resistant than the standard strain of *E. coli.* (Table VI).

In some instances marked variations occurred in the sensitivity of the various strains within a single species. (Table VII). Likewise, there were at times marked differences in the sensitivity of individual organisms within a single strain. (Table VIII).

Comparison of Sensitivity to Crude and

TABLE IX.
Comparison of Bacteriostatic Action of Crude and Purified Streptomycin *in Vitro.*

Strain	Sensitivity in μg per cc*				
	Crude sulfate 453 μg/mg	Pure sulfate 802 μg/mg	Crystalline (CaCl$_2$ double salt)		
			Prep. 1 685 μg/mg	Prep. 2 701 μg/mg	Prep. 3 708 μg/mg
B. subtilis (W)	0.14	0.25	0.25	0.19	0.11
K. pneumoniae	0.17	0.16			
B. mycoides (W)	0.21	0.25	0.25	0.25	0.17
A. aerogenes	0.33	0.29	0.25	0.19	0.22
” ”	0.52	0.32			
E. coli (W)	1.00	1.00	1.00	1.00	1.00
Staph. aur. (H)	2.20	2.50	2.50	2.50	2.00
Strep. hem. (C203Mv)	2.40	2.10			
E. typhosa	5.70	4.00	>5.00	5.00	>5.00

* Sensitivity = ratio of least amount of streptomycin (μg per cc) causing complete inhibition of growth of unknown strain to least amount (μg per cc) causing inhibition of standard strain of *E. coli* on same day.

Pure Streptomycin. The sensitivity of a small group of organisms against a partially purified preparation of streptomycin sulfate (potency, 453 μg per mg) was compared with their sensitivity to 3 preparations of the crystalline CaCl$_2$ double salt of streptomycin and to one preparation of pure streptomycin sulfate prepared from a crystalline salt. The difference in sensitivity was insignificant and it is apparent that the strains tested were equally sensitive to the preparations of impure and pure streptomycin used. (Table IX).

Subsequent experiments using different preparations of impure streptomycin revealed a somewhat different situation however. Using 5 different strains of *E. typhosa* as test organisms, the sensitivity to 2 preparations of impure streptomycin sulfate and to one preparation of highly purified streptomycin sulfate was compared. The sensitivity of all 6 strains to the preparation of highly purified streptomycin sulfate was >36 μg per cc. Likewise the sensitivity to one preparation of impure streptomycin sulfate ranged from 24 to 36 μg per cc. However the sensitivity to the other preparation of impure streptomycin sulfate ranged from 3.3 to 7.0 μg per cc. It was apparent that some preparations of impure streptomycin sulfate were more effective than others against *E. typhosa.* (Table X).

TABLE X.
Comparison of Bacteriostatic Action of Crude and Purified Streptomycin Against *E. Typhosa in Vitro.*

Strain	Sensitivity in μg/cc (72 hr)*		
	Pure sulfate 802 μg/mg	Impure sulfate	
		Prep. 1 130 μg/mg	Prep. 2 453 μg/mg
E. typhosa (stock)	>40		32
(Ga)	>40	4.3	36
(Me)	>36	3.3	24
(Mc)	>40	7.0	24
(Ur)	>36	3.8	40

* Sensitivity expressed in terms of least amount of streptomycin (μg per cc) necessary to cause inhibition of growth.

Discussion. The sensitivity of an organism to streptomycin is influenced by many factors including (1) age of culture, (2) concentration of organisms, (3) growth phase of the culture, and (4) constituents of the medium used. Provided the age, concentration and growth phase of the organisms are constant, sensitivities will remain constant from day to day if the same medium is used throughout. Since differences in medium alter so greatly the sensitivity of an organism, comparison of sensitivities is facilitated by expressing them in relation to a standard strain tested simultaneously in the same medium.

The effect of streptomycin is highly bacteriostatic. Only against a few organisms (*H. influenzae, Pasteurella tularensis, A. aerogenes*) has bactericidal activity been demonstrated.[8] The fact that with many strains the number of organisms in relation to the concentration of drug so greatly influences the effect of the streptomycin is probably a limiting factor in its usefulness. In like manner the effectiveness of the sulfonamides has been dependent at least in part on the concentration of organisms in relation to the amount of sulfonamide present. The number of units of penicillin necessary for inhibition of a culture, on the other hand, is altered only by large variations in the number of organisms present.

One can only speculate on the relationship this may have to the apparent development of bacterial resistance to streptomycin. It is now recognized however that in contrast to penicillin, the effectiveness of streptomycin *in vivo* is at times limited by the ease with which susceptible strains of microorganisms develop resistance to it.

Under the experimental conditions used, no differences have been demonstrated in the sensitivity of most organisms to impure streptomycin sulfate, to the crystalline $CaCl_2$ double salt of streptomycin, or to highly purified streptomycin sulfate prepared from a crystalline salt. Impure streptomycin and highly purified or crystalline streptomycin appear equally effective *in vitro* against all organisms except *E. typhosa*. The significance of the fact that certain preparations of impure streptomycin sulfate are more effective against strains of *E. typhosa* than highly purified streptomycin will be discussed in detail elsewhere.

Summary. 1. A standardized procedure for determination of sensitivity of microorganisms to streptomycin is described.

2. The sensitivity of an organism to streptomycin is influenced by age of culture, concentration of organisms, growth phase of culture, and constituents of medium used. Providing these factors are held constant, the sensitivity of a given strain will remain constant from day to day.

3. The action of streptomycin is bacteriostatic rather than bactericidal. Its action is inhibited by certain growth stimulating substances such as peptone, as well as by certain reducing substances.

4. The sensitivity of 84 strains belonging to 7 species is described. Marked variation in sensitivity exists between different strains within a single species and at times between different cells within a given strain.

5. The sensitivity of 9 strains belonging to 8 species is essentially the same when tested against crude streptomycin sulfate (453 μg/mg), against 3 preparations of the crystalline $CaCl_2$ double salt of streptomycin (685 to 708 μg/mg) and against a preparation of streptomycin sulfate (802 μg/mg) prepared from a crystalline salt.

6. The sensitivity of 4 strains of *E. typhosa* to certain preparations of impure streptomycin sulfate is greater than to highly purified streptomycin sulfate.

15923

Observations on the Action of Streptomycin *in vitro* (II).*

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It is recognized that a wide variety of factors may influence the observed sensitivity of an organism to streptomycin. Certain of these factors have been discussed in a recent communication from this laboratory.[1] The sensitivity of a number of strains belonging to 10 different species was described and it was shown that with the exception of *E. typhosa* the strains tested were equally sensitive to impure streptomycin sulfate, to highly purified streptomycin sulfate and to the crystalline $CaCl_2$ double salt of streptomycin.

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* A part of this work was presented at the Conference on Antibiotic Research held at Washington, D.C. on January 31 and February 1, 1947 under the auspices of the Antibiotics Study Section of the National Institute of Health.

[1] Lenert, T. F., and Hobby, G. L., Proc. Soc. Exp. Biol. and Med., 1947, **65**, 235.

The differences in sensitivity of an organism caused by alteration in medium, or variation of culture from day to day, are no greater than the difference in sensitivity of a strain when tested against various preparations on the same day or the difference in sensitivity of individual cells within a given strain.[1] An actual increase in the resistance of strains of bacteria to streptomycin both *in vitro* and *in vivo* has been observed by many investigators, however.[2–11]

Experimental. It has been amply demonstrated that the presence of serum alters the sensitivity of an organism to penicillin.[12–15] Since the chemotherapeutic effect of any such agent is dependent upon its activity in the presence of body fluids and since the demonstration of blood or serum levels is dependent upon the determination of the sensitivity of a standard organism in the presence of serum, the present study was undertaken to determine the effect of serum upon the sensitivity of various organisms to streptomycin. In addition, the effect of certain related factors have been investigated.

Effect of Serum on Sensitivity of Organisms to Streptomycin. Pooled normal human serum and normal horse serum were used throughout. The sensitivity of a number of organisms belonging to several species was determined against a preparation of streptomycin sulfate[†] having a potency of 802 μg per mg. The procedure used was identical with that previously described[1] except that broth containing varying amounts of serum was used in place of plain broth.

A total of 31 strains belonging to 7 different species were tested in broth containing 0, 1, 5, 10, 20 and 50% normal horse serum. Thirteen strains, also belonging to 7 different species, were tested in broth containing the same amounts of human serum.

The sensitivity of the Gram negative organisms tested (i.e., *E. coli, E. typhosa, K. pneumoniae,* and *A. aerogenes*) was unaltered by addition of 1 to 5% of either human or horse serum to the medium. Larger amounts of serum increased the sensitivity of these strains slightly. Certain strains of *Staphylococcus aureus* were similarly affected by serum whereas others became considerably less sensitive to streptomycin when concentrations of 1 to 50% serum were present. All strains of *Streptococcus hemolyticus, Streptococcus viridans,* and *Diplococcus pneumoniae* showed a marked increase in resistance to streptomycin in the presence of 1 to 5% serum. (Tables I, II).

The average sensitivity for each species of organisms tested again illustrates the marked increase in resistance of the Gram positive organisms. (Graph I).

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[†] All streptomycin used throughout this study was prepared by Chas. Pfizer and Co.

TABLE I.

Effect of Horse Serum on Sensitivity of Organisms to Streptomycin.

	Sensitivity in μg per cc. % serum					
Strain	0	1	5	10	20	50
E. coli						
(W)	5.4	4.4	4.5	4.4	3.6	1.5
(B)	3.0	3.0	4.0	3.0	2.0	1.0
(T)	7.0	3.0	6.0	4.0	3.0	2.0
(Y)	4.0	3.0	4.0	3.0	3.0	<1.0
(A)	4.0	3.0	4.0	3.0	3.0	<1.0
(H)	4.0	3.0	4.0	4.0	3.0	2.0
E. typhosa						
(S)	32.0	24.0	24.0	32.0	32.0	<8.0
(Ga)	36.0	36.0	(20.0)	28.0	16.0	8.0
(M)	32.0	32.0	28.0	24.0	20.0	16.0
(Mc)	>40.0	>40.0	40.0	36.0	24.0	8.0
(U)	28.0	32.0	36.0	36.0	32.0	8.0
(G)	>40.0	40.0	32.0	36.0	32.0	12.0
A. aerogenes	1.5	1.5	1.25	1.25	1.0	1.0
Staph. aur.						
(H)	<4.0	20.0		20.0	8.0	<4.0
(T)	2.0	6.0	5.0	4.0	3.0	3.0
(HT)	2.0	2.0	4.0	3.0	2.0	<1.0
(HS)	4.0	5.0	6.0	4.0	3.0	2.0
(M)	2.0	6.0	4.0	2.0	3.0	<1.0
(K)	<1.0	5.0	5.0	3.0	3.0	3.0
(HS$_2$)	24.0	24.0	24.0	40.0	32.0	16.0
D. pneumoniae						
(I/230)	4.0	36.0	28.0	32.0	36.0	
(D/39)	4.0	16.0	28.0	20.0	28.0	
(A66)	<1.0	20.0	20.0	24.0	28.0	
Strep. hem.						
(C203Mv)	<4.0	36.0	40.0	>40.0	40.0	36.0
(Ch)	<4.0	20.0	24.0	24.0	24.0	16.0
(090)	10.0	>50.0	>50.0	>50.0	>50.0	45.0
(H$_{76}$)	<5.0	40.0	40.0	40.0	45.0	35.0
(C$_1$)	20.0	>50.0	>50.0	>50.0	>50.0	>50.0
Strep. vir.						
(R)	32.0	>80.0	>80.0	>80.0	>80.0	>80.0
(T)	15.0	>50.0	>50.0	50.0	45.0	35.0
(L)	30.0	>50.0	>50.0	>50.0	>50.0	>50.0

Sensitivity = least amount of streptomycin causing complete inhibition of growth after 72 hours at 37°C.

Strains C203Mv and "Ch" belong to the Lancefield Serological Group A; Strain 090 to Group B; Strain H$_{76}$ to Group C; Strain C$_1$ to Group D.

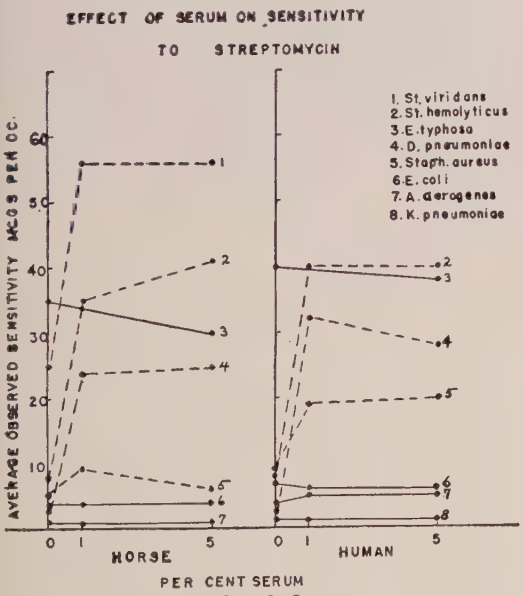
Effect of Serum on Growth of Organisms with and without Streptomycin. In view of the possibility that the decrease in the sensitivity of the Gram positive organisms to streptomycin in the presence of serum might be merely a reflection of their greater ability to multiply in the presence of serum, further experiments were carried out to determine the effect of serum on 3 representative strains: (1) a standard strain of *E. coli* (W), (2) the Oxford strain of *Staphylococcus aureus* (Strain H), and (3) *Streptococcus hemolyticus*, strain C203Mv.

Streptomycin sulfate having a potency of 802 μg per mg was used throughout this experiment. To each of 6 tubes was added broth containing 0, 1, 5, 10, 20 and 50% pooled human serum. To each tube, sufficient streptomycin was added to give a final concentration of 2 μg per cc. Each tube was

TABLE II.
Effect of Human Serum on Sensitivity of Organisms to Streptomycin.

Strain	Sensitivity in μg per cc % serum 0	1	5	10	20	50
E. coli						
(W)	8.5	8.0	8.5	6.0	4.5	3.0
(Y)	5.0	5.0	4.0	4.0	4.0	2.0
(H)	7.0	7.0	6.0	5.0	4.0	2.0
E. typhosa						
(S)	>40.0	>40.0	>40.0	>40.0	32.0	8.0
(Ga)	40.0	20.0	32.0	36.0	32.0	12.0
(U)	>40.0	32.0	>40.0	24.0	28.0	8.0
A. aerogenes	2.0	2.5	2.0	2.0	1.5	1.0
K. pneumoniae	1.0	1.0	1.0	<0.5	<0.5	<0.5
Staph. aur.						
(H)	16.0	>40.0	>40.0	40.0	40.0	20.0
(M)	5.0	7.0	10.0	5.0	3.0	3.0
(HS)	8.0	>10.0	10.0	>10.0	8.0	4.0
Strep. hem. (C203Mv)	8.0	>40.0	>40.0	>40.0	>40.0	>40.0
D. pneumoniae						
(I/230)	4.0	36.0	40.0	32.0	32.0	16.0
(D/39)	4.0	20.0	24.0	20.0	20.0	8.0
(A66)	<1.0	40.0	20.0	24.0	20.0	8.0

Sensitivity = least amount of streptomycin causing complete inhibition of growth after 72 hours at 37°C.

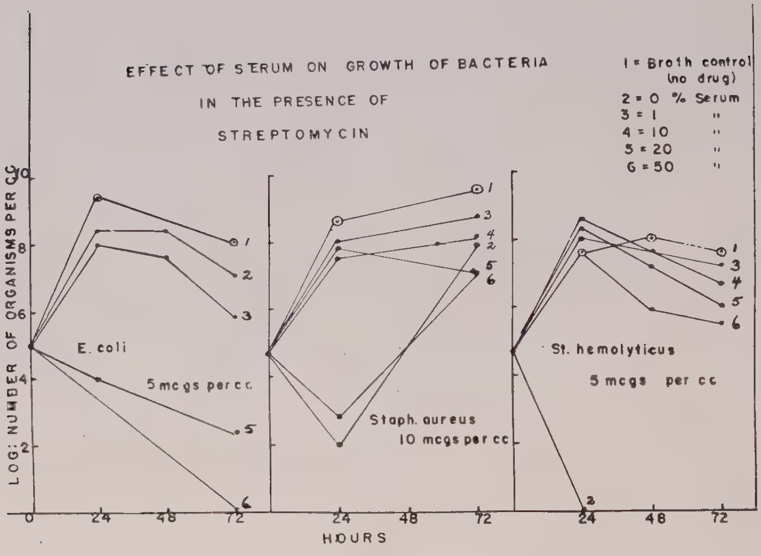


Graph I.

seeded with a sufficient amount of a 6 hour plain broth culture of *E. coli* (W) to give a final dilution of 1:2000. Prior to dilution, the culture had been adjusted to 78% transmission, Photovolt lumetron No. 400. The number of organisms per cc was determined by plate counts at 0, 24, 48, and 72 hours. Similarly, *E. coli* was tested using 5 μg of streptomycin per cc, *Staphylococcus aureus* (H) was tested using 5, 10, and 20 μg per cc and *Streptococcus hemolyticus* (C203Mv) using 2, 5, and 40 μg per cc. Similar tests were carried out on each organism using horse serum.

In the case of *E. coli*, there was no evidence that growth was altered by the presence of 1, 20 or 50% horse or human serum. A concentration of 2 μg of streptomycin caused no inhibition of growth either in plain broth or in serum broth. Five micrograms of streptomycin per cc, on the other hand, produced a slight bacteriostatic effect in plain broth. Its action was greatly enhanced in the presence of 20 to 50% human serum or 50% horse serum. (Graph II).

The growth of *Staphylococcus aureus* (strain H) was likewise unaffected by con-



Graph II.

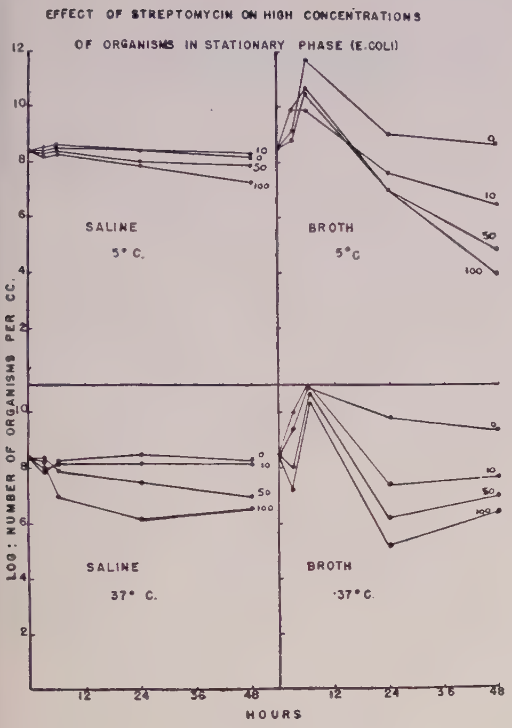
centrations of 1 to 50% horse or human serum. In the presence of 5 μg of streptomycin per cc, there was no bacteriostatic action either in the presence or absence of serum. In the presence of 10 or 20 μg of streptomycin per cc of plain broth, temporary bacteriostasis occurred. This bacteriostatic action was completely inhibited by 1, 10 or 20% human or horse serum, whereas the effect in 50% serum was comparable with that in plain broth. (Graph II).

The growth of *Streptococcus hemolyticus* was most markedly affected by the presence of serum. Concentrations of 1 to 50% serum caused a slight but definite increase in the rate of growth. In the presence of 2 μg of streptomycin per cc of plain broth, temporary bacteriostasis occurred whereas 5 μg per cc was adequate to produce sterilization of the culture within 24 hours. This effect was completely inhibited by 1, 10, 20 or 50% horse or human serum. The bactericidal action of 40 μg of streptomycin per cc was likewise completely inhibited by 1, 10, and 20% horse or human serum. (Graph II).

These results are comparable with those indicated by the alteration in sensitivity of organisms to streptomycin, in the presence of serum. The effect of serum on the sensitivity of an organism is greater than any effect of serum on the rate of growth of the organism, as shown by growth curves.

Effect of Streptomycin on Stationary or Slowly Dividing Cells. In view of the possible correlation between rate of growth and the effect of serum on the sensitivity of various organisms to streptomycin, experiments were carried out to determine the effect of streptomycin on stationary or slowly dividing cells and on rapidly dividing cells.

An 18 hour broth culture of *E. coli* was centrifuged and the organisms were resuspended (1) in saline and (2) in broth. Each suspension was divided into 4 parts and to 3 of each sufficient streptomycin was added to give final concentrations of 10, 50, and 100 μg per cc respectively. The fourth tube from each series was held as control. The saline suspensions were incubated at 5°C. Similar suspensions were prepared and incubated at 37°C. Colony counts were made at 3, 6, 24, and 48 hours. No multiplication took place in saline at either 5°C. or 37°C. Streptomycin in a concentration of 10 μg per cc exerted no action against the organisms present. In the presence of 50 and 100 μg of streptomycin per cc, a slight decrease in the number of organisms occurred at 37°C. after a lag of from 3 to 6 hours. The effect at 5°C. was not significant. In broth the number of organisms increased many fold. A slow bactericidal action was observed with 10 as well as with 50 and 100 μg per cc of streptomycin. The decrease in the number

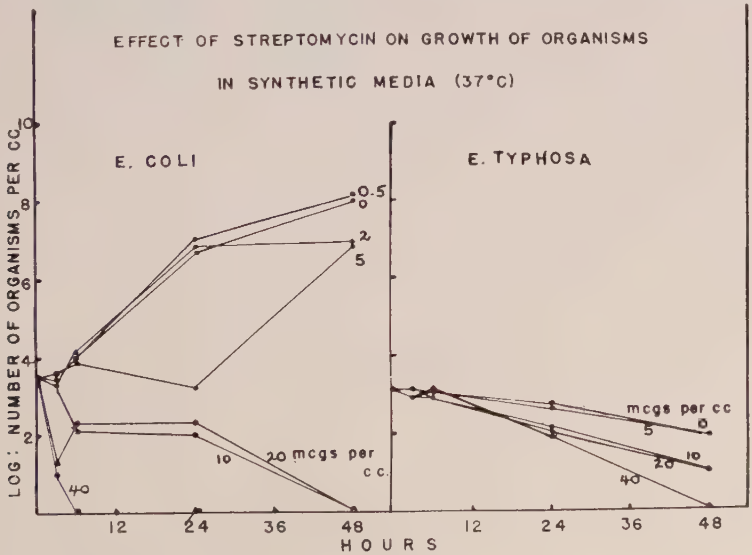


Graph III.

of organisms occurred only after a lag of at least 6 hours during which time definite multiplication took place. The decrease in broth was far greater than that in saline. It was apparent that under certain conditions streptomycin is capable of acting on both stationary and dividing cells provided a sufficiently high concentration of drug is used. The effect is greater on dividing cells however. (Graph III).

The difference in the action of streptomycin on rapidly dividing cells and on slowly dividing or resting cells was further demonstrated in the following typical experiment. McLeod's synthetic medium containing asparagin and no glucose was prepared in such a manner as to contain 0, 0.5, 2, 5, 10, 20 and 40 μg of streptomycin per cc. Two tubes of each concentration were set up; one was inoculated with a sufficient amount of an 18 hour plain broth culture of *E. coli* to yield a final dilution of 10^{-5}, the other was similarly inoculated with *E. typhosa*. In each case, the cultures used were centrifuged and washed 3 times in the synthetic medium prior to dilution. Incubation was carried out at 37°C. and the number of organisms per cc determined by plate counts at 0, 3, 6, 24, and 48 hours.

E. coli multiplied rapidly in the synthetic medium. Likewise, in the presence of 0.5 and 2.0 μg of streptomycin per cc, rapid multiplication took place. A temporary bacteriostatic action occurred during the first 24 hours of incubation in the presence of 5 μg per cc. With larger amounts of streptomycin, there was a definite and permanent



Graph IV.

decrease in the number of organisms.

E. typhosa showed no growth in this synthetic medium. The number of organisms per cc remained constant. In the presence of 2.0 and 5.0 μg of streptomycin per cc, the number of organisms also remained constant. With 10, 20, or 40 μg per cc, however, there was a slight and gradual decrease in the number of organisms. (Graph IV).

It was again apparent that streptomycin, if in sufficient concentration, can act at 37°C. on slowly dividing or stationary cells. In view of the large initial number of organisms present in these experiments and the sensitivity of the strains used, the concentrations of streptomycin necessary to demonstrate bacteriostatic action were not exceptionally high, however.

Discussion. The relation of number of organisms and concentration of drug to the sensitivity of an organism to streptomycin has been discussed elsewhere. It is recognized that, in contrast to penicillin, the effectiveness *in vivo* is limited by the fact that susceptible strains of microorganisms frequently develop resistance to it. Undoubtedly, many factors influence this development of resistance. It has been suggested by Reimann and his coworkers that certain factors in the body may interfere with the bacteriostatic action of streptomycin.[16,17] Wolensky and Steenken[8] on the other hand were unable to demonstrate any destruction of streptomycin or any alteration in its bacteriostatic or bactericidal power by contact with broth, serous body fluids, pus or normal tissue juices.

In the present study, data have been presented which further suggest that factors within the body may enhance development of resistance. It has been demonstrated that serum markedly increases the resistance of certain of the Gram positive organisms to streptomycin. The effect of other body fluids or tissue substances on these or other bacterial species remains to be determined. It is obvious however that the sensitivity of an organism to streptomycin, as determined *in vitro* in the usual broth medium, is not necessarily an index of the *in vivo* sensitivity of the organism.

The importance of serum in the bioassay determination of penicillin in blood has been amply demonstrated by Tompsett, Schultz, and McDermott[15] and by Richardson and his associates.[18] Since, of necessity, serum is present in all such determinations, and since the effect is so marked in the case of streptomycin, it is apparent that the organism used in such assays must be one that is affected little if any by the concentration of serum present.

Whether the alteration in sensitivity to streptomycin, induced by the presence of serum, is due to an actual change in the organism or to the outgrowth of resistant forms already present cannot be determined from the data available here. That the effect is not due to an actual destruction of streptomycin by serum is suggested by growth curves and by the fact that the sensitivity of many organisms is unaltered in the presence of serum. It seems more likely that the apparent resistance to streptomycin is due to an alteration in the metabolic processes of certain bacterial species when grown in serum.

Summary. 1. The presence of 1 to 5% or more horse or human serum enhances the resistance of *Streptococcus hemolyticus*, *Streptococcus viridans*, *Diplococcus pneumoniae*, and certain strains of *Staphylococcus aureus*.

2. Serum in concentrations of 1 to 5% has no effect on the sensitivity of *E. coli*, *E. typhosa*, *K. pneumoniae*, or *Aer. aerogenes* to streptomycin. Higher concentrations of serum may at times enhance the action of streptomycin on certain of these organisms.

3. Streptomycin is effective under certain conditions against stationary or slowly dividing cells, as well as against rapidly dividing cells, provided a sufficient concentration of the drug is used.

4. No direct correlation between the rate

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[16] Reimann, H. A., Price, A. H., and Elias, W. F., *Arch. Interm. Med.*, 1945, **76**, 269.

[17] Elias, W. F., and Durso, J., *Science*, 1945, **101**, 589.

[18] Richardson, A. P., Miller, I., Schumacher, C., Jambar, W., Pansy, F., and Lapedes, D., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 514.

of growth of an organism with or without serum and the effect of serum on the sensitivity of an organism to streptomycin can be made at this time.

15924

Biological Activity of a Residual Form of Streptomycin against *Eberthella typhosa.*[*]

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The fact that a number of naturally occurring forms of penicillin have been recognized[1] suggests that other antibacterial agents of biologic origin may also occur in more than one form. The antibacterial action of impure and highly purified streptomycin sulfate and of the crystalline $CaCl_2$ double salt of streptomycin is discussed in detail elsewhere.[2,3] Under the experimental conditions used, a slight difference in their activity was apparent against *E. typhosa* but not against the other organisms tested.

Experimental. In the preparation of a crystalline salt of streptomycin from partially purified material (400 to 500 µg per mg), a residual fraction remains. This fraction possesses antibacterial activity. The present report covers preliminary observations which demonstrate that in certain biological aspects it differs from the impure or highly purified streptomycin sulfate,[†] as produced commercially, and from the crystalline $CaCl_2$ double salt of streptomycin.

Bacterial Spectrum. A comparison was made of the sensitivity of 7 different organisms to the residual form of streptomycin, to impure streptomycin sulfate, and to highly purified streptomycin sulfate prepared from a crystalline salt. The procedure used was identical with that described in a previous communication.[2] Sensitivity has been accepted throughout as the least amount of streptomycin in µg per cc causing inhibition of growth as evidenced by absence of gross turbidity at the end of 72 hours incubation at 37°C.

No significant differences in the sensitivity of *Aer. aerogenes, E. coli, Bc. subtilis* or *Staphylococcus aureus* to these streptomycins was observed. The difference in the sensitivity of *E. typhosa* however was marked. Whereas 40 or more µg of impure or highly purified streptomycin sulfate per cc were necessary for inhibition of this strain, only 8 µg of the crude residue were necessary (Table I).

Other preparations of streptomycin residue

TABLE I.
Bacterial Spectrum of 3 Forms of Streptomycin.

	Sensitivity in µg per cc		
Strain	Crude sulfate 453 µg/mg	Pure sulfate 802 µg/mg	Residue 165 µg/mg
A. aerogenes	2.	2.	2.
B. subtilis (W)	1.5	1.	1.
B. mycoides (W)	1.5	1.5	2.
E. coli (W)	6.	7.	7.
Staph. aur. (H)	20.	18.	18.
E. typhosa	40.	>40.	8.
M. tuberculosis (H$_{37}$Rv)	5.	5.	5.

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[*] A part of this work was presented at the Conference on Antibiotic Research held at Washington, D.C. on January 31 and February 1, 1947 under the auspices of the Antibiotics Study Section of the National Institute of Health.

[1] Report by the Committee on Medical Research, O.S.R.D. Washington, and the Medical Research Council, London, on Chemistry of Penicillin, *Science*, 1945, **102** (2660), 627.

[2] Lenert, T. F., and Hobby, G. L., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 235.

[3] Hobby, G. L., and Lenert, T. F., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 242.

[†] All streptomycin used throughout this study was prepared by Chas. Pfizer and Co.

TABLE II.
Bacterial Spectrum of Impure Streptomycin Residue as Compared to Impure and Purified Streptomycin Sulfate.

Streptomycin	Potency in μg/mg	Strain: Sensitivity in μg per mg A. aerog.	*E. coli*	*Staph. aur.*	*E. typh.*
Pure sulfate (standard)	802	2.25	7.5	19	32
Impure sulfate	277	5	7	20	20
Impure residue					
Prep. No. 1	187	1.5	7	20	8
,, 2	40	2	6	>20	<6
,, 3	124	2	5	20	8
,, 4	43	2	6	>20	<6
,, 5	68	3	6	>20	<6
,, 6	35	3	8	>20	<4

have been tested and found to show the same effect (Table II).

Four freshly isolated strains and one additional stock strain of *E. typhosa* were similarly tested for sensitivity to preparations of impure streptomycin sulfate, highly purified streptomycin sulfate prepared from crystalline material, and to the crude streptomycin residue. All 5 strains were at least 2 to 5 times more sensitive to the streptomycin residue than to highly purified streptomycin sulfate. The sensitivity to impure streptomycin sulfate was less than the sensitivity to the streptomycin residue although greater than the sensitivity to the highly purified sulfate preparations. The relative order of sensitivity to the 3 forms was consistent with the fact that they represent successive steps of purification (Table III).

A single preparation of streptomycin residue was partially purified and the resultant active fraction again tested against *Aer. aerogenes*, *E. coli*, *Staphylococcus aureus*, and *E. typhosa*. All of the strains tested were more sensitive to this partially purified residue than they were to the crystalline salt. The difference was most marked, however, with *E. typhosa* (Table IV).

TABLE III.
Action of Various Forms of Streptomycin on *E. typhosa*.

Strain		Sensitivity in μg per cc Crude sulfate 453 μg/mg	Pure sulfate 802 μg/mg	Residue 165 μg/mg
E. typhosa	(stock)	40	>40	8
	(Ga)	36	>40	8
	(Me)	24	>40	16
	(Mc)	24	>40	8
	(Ur)	40	>40	20

TABLE IV.
Comparison of Bacterial Spectrum of Streptomycin Residue and Crystalline $CaCl_2$ Double Salt of Streptomycin.

Strain	Sensitivity in μg per cc Original residue 165 μg/mg	Partially purified residue 22000 μg/cc	Crystalline double salt 780 μg/mg
A. aerogenes	2.5	1.5	2.5
E. coli	>10	7	>10
Staph. aur. (H)	20	16	>20
E. typhosa	8	8	>40
M. tuberculosis ($H_{37}Rv$)	5	5*	

* No apparent difference in sensitivity as indicated by the No. of μg per cc necessary for complete inhibition of growth. In the presence of 5 μg/cc of the partially purified residue, however, growth appeared much more slowly and to a less extent than in the presence of 5 μg per cc of purified streptomycin sulfate, crude streptomycin sulfate or the original residue. All sensitivity determinations on *M. tuberculosis* were carried out in the Dubos medium.[4]

Antibacterial Action on Organisms Grown on Solid Media. Recognition of the various penicillins as distinct entities was facilitated by the early observation of Coghill[5] that the degree of antibacterial activity of these substances differed when tested against *Bc. subtilis* and against *Staphylococcus aureus* by the Oxford cup plate method. In view of the fact that the streptomycin residues described above possessed such marked activity against *E. typhosa,* as compared to impure streptomycin sulfate or highly purified streptomycin sulfate prepared from crystalline material, it seemed likely that a similar difference could be detected by the Oxford method.

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[4] Dubos, R. J., and Davis, B. D., *J. Exp. Med.*, 1946, **83**, 409.

[5] Schmidt, W. H., Ward, G. E., and Coghill, R. D., *J. Bact.*, 1945, **49**, 411.

Using as standard a preparation of highly purified streptomycin sulfate, having a potency of 802 μg per mg and prepared from a crystalline salt, the potency of a number of preparations of the streptomycin residue was determined against *E. typhosa.* All of these residues showed a potency per mg against *E. typhosa* considerably higher than the official *Bc. subtilis-E. coli* value.‡ The *E. typhosa-Bc. subtilis (E. coli)* differential ratios of these preparations of streptomycin residue ranged from 1.94 to 3.14. All of these preparations were highly impure (Table V).

Effect of Various Forms of Streptomycin on Growth of Organisms in Vitro. The difference in the activity of the residual form of streptomycin as compared to the impure or purified streptomycin sulfate was further demonstrated in growth curves on *E. coli, E. typhosa,* and *Streptococcus hemolyticus.* Eighteen-hour broth cultures diluted in streptomycin broth immediately prior to use to give a concentration of 1:2000 organisms per cc were used. The concentrations of streptomycin used represented amounts slightly below and slightly above the sensitivity of the individual strain to the specific form of streptomycin. Incubation was carried out at 37°C. The number of organisms per cc was determined by plate counts at 0, 24, 48, and 72 hours.

TABLE V.
Activity of Streptomycin Residue Oxford Assay Method.

Sample No.	Potency (units per mg)		*E. typh.-B. subt.** ratio
	*B. subt.-E. coli**	*E. typhosa*	
1	165	363	2.20
2	210	407	1.94
3	40	95	2.38
4	124	283	2.28
5	43	135	3.14
Standard: Streptomycin sulfate	802	802	1.00

* Assay values with *B. subtilis* and *E. coli* are similar. Average of these potencies used throughout.

No definite difference could be detected in the effect of the 3 forms of streptomycin on the growth of *E. coli* or *Streptococcus hemolyticus.*

In the case of *E. typhosa,* the difference was marked. Fifteen μg of the crude residue per cc showed greater antibacterial activity against this organism than 30 μg of impure streptomycin sulfate or 50 μg of pure streptomycin sulfate per cc.

These results were consistent with the fact that the sensitivity of this organism to the crude residue was only 8 μg per cc whereas its sensitivity to the impure streptomycin sulfate was 40 μg per cc and to the purified streptomycin sulfate, >40 μg per cc (Graph I).

Effect of Serum on the Sensitivity of Organisms to the Residual Streptomycin. In a previous communication,[3] the effect of serum on the sensitivity of certain organisms to impure and highly purified streptomycin sulfate was described. It was shown that concentrations of 1 to 5% horse or human serum decreased the sensitivity of certain of the Gram-positive organisms to these forms of streptomycin.

Similar experiments were carried out to determine the effect of serum on the sensitivity of 6 different organisms to the streptomycin residue. Concentrations of 1, 10, 20 and 50% pooled normal human serum were used. The high buffering capacity of the broth medium served to maintain a constant pH of 7.8 throughout.

The resistance of the streptococcus and

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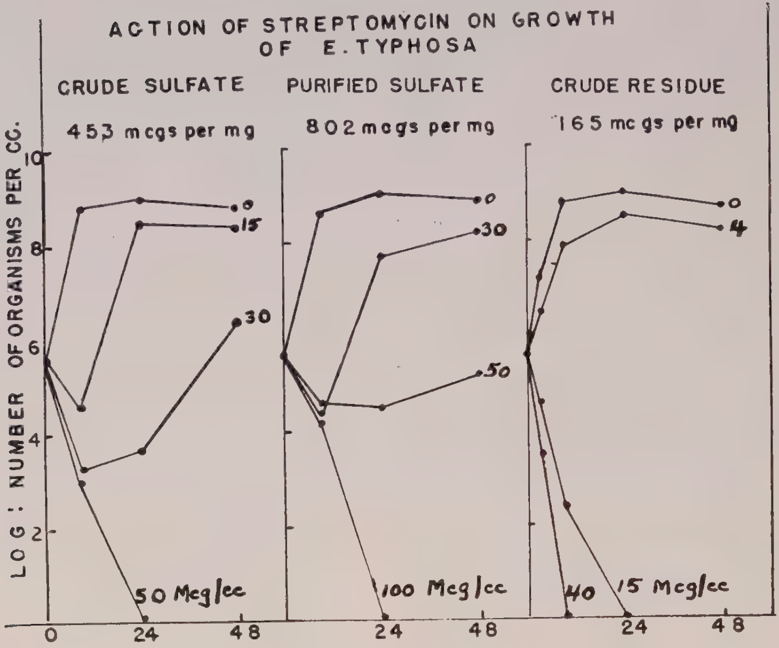
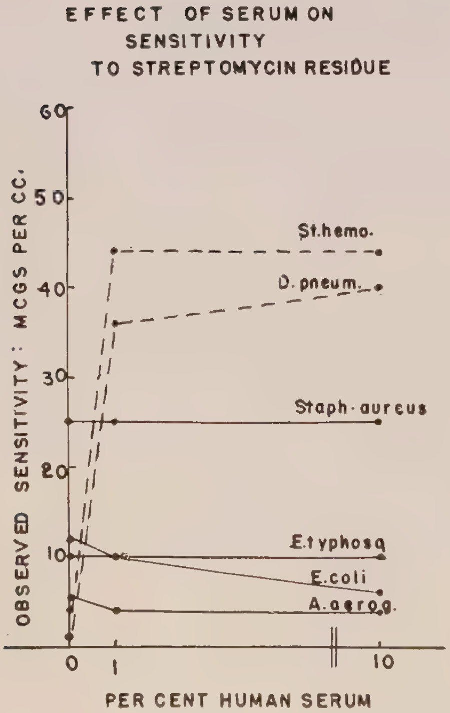
‡ The potency of streptomycin in terms of micrograms of streptomycin per mg of material is determined routinely according to the procedure recommended by the Food and Drug Administration. The Oxford cup plate method, using *Bacillus subtilis* as the test organism, is the official test. Similar results may be obtained by the turbidimetric method, using *E. coli* as the test organism, and the average on the 2 tests has been used in the present study.



Graph I.



Graph II.

pneumococcus strains tested was greatly increased whereas the single strain of staphylococcus tested reacted similarly to the Gram-negative strains whose sensitivity was unaltered (Graph II).

The effect of serum on the sensitivity of various organisms to the streptomycin residue was thus identical with its effect on their sensitivity to impure or highly purified streptomycin sulfate as previously reported.[3]

Effect of Residual Streptomycin in E. Typhosa Infections in Mice. A stock strain of *E. typhosa* was used as the infecting organism throughout these experiments. One cc of 10^{-2}, 10^{-3}, and 10^{-4} dilutions of a 16-hour plain broth culture, diluted in 5% mucin according to the method of Miller,[6] was injected intraperitoneally into each of a series of 20 g white mice. A minimum of 8 to 10 mice was used for each dilution of each series. Treatment was started 2 hours after the infecting dose. The streptomycin was administered to part of the animals subcutaneously in peanut oil[7] in divided dosage. To the remainder of the animals it was administered in aqueous solution by mouth.

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[6] Miller, C. P., Proc. Soc. Exp. Biol. and Med., 1935, **32**, 1136.

[7] Hobby, G. L., Meyer, K., and Chaffee, E., Proc. Soc. Exp. Biol. and Med., 1942, **50**, 277.

TABLE VI.
Chemotherapeutic Action of Various Forms of Streptomycin on *E. typhosa* Infections in Mice.

Form of streptomycin		Impure sulfate	Crude residue
Potency: µg per mg		802	165
Ratio: $\frac{\textit{E. typhosa}}{\textit{Bc. subt.-E. coli}}$		1.00	2.20
Route of administration*	Total dosage† µg	Therapeutic effect (% survival)	
Subcutaneous	60	21.7	26.1
	110	47.9	40.0
	175	45.8	35.0
	240	64.6	80.6
	500	80.8	78.2
Oral	100	10.7	10.7
	250	19.2	7.3
	500	37.9	16.6
Controls	0	15.3	

* All mice were infected by the intraperitoneal route; treatment was carried out by the subcutaneous or oral routes.

† 10^{-2}, 10^{-3}, and 10^{-4} dilutions of culture and a minimum of 8-10 mice per dilution were used for each dosage level tested.

Forty per cent of the total dose was administered 2 hours, 40% 7 hours, and 20% 24 hours after infection. Total dosages of 60, 110, 175, 240 and 500 µg were used in the subcutaneously-treated animals; 100, 250, and 500 µg in the animals treated by mouth. A control series of untreated animals was included in each experiment. The untreated infection uniformly produced death in 85% of animals. The results may be seen in Table VI. The streptomycin residue was active *in vivo* when administered by the intraperitoneal route. Little or no difference in the protective power of this material and of the purified streptomycin sulfate was observed. The conditions of the experiment, however, and the nature of the infection in mice may not have been such as would show a quantitative difference in the activity of these fractions. By the oral route no protection resulted from the administration of 100, 250, or 500 µg of either form of streptomycin.

Absorption of Residual Streptomycin. A comparison of the absorption of a single preparation of streptomycin residue, and of impure and highly purified streptomycin was carried out in a small series of rabbits following the intramuscular injection of 5000 µg per kg of body weight. The concentration of streptomycin per cc of serum was determined at 0, 1, 2, 3, 4, and 6 hours by the Oxford cup plate method using *B. subtilis* as the test organism. It is recognized that the rabbit is an unsatisfactory animal for absorption and excretion studies. Serum levels varied widely from one animal to another. The average levels on 5 to 6 animals per sample, however, showed no significant difference in the rate at which impure streptomycin sulfate, highly purified streptomycin sulfate, or crude streptomycin residue is eliminated from the blood stream.

Discussion. A residual form of streptomycin, which is a more efficient antibacterial agent against *E. typhosa in vitro,* has been described. The sensitivity of the typhoid bacillus to this residual fraction of streptomycin obtained during the purification process is at least 2 to 5 times greater than to the crystalline $CaCl_2$ double salt of streptomycin. By the Oxford cup plate method, the *E. typhosa-Bc. subtilis (E. coli)* differential ratio of impure preparations of this material is approximately 2.0-3.0.

The significance of the active material present in the streptomycin residue remains to be determined. Whether or not this residual substance is actually a different streptomycin than that with which we are familiar at present must await further chemical separation and chemical analysis. Fried and Titus[8] have recently described a form of streptomycin (B) differing in *in vitro* activity from purified streptomycin. Furthermore, present evidence suggests that crystalline streptomycin, as prepared today, may not be entirely uniform.

In the case of penicillin, 5 naturally occurring forms have been described. These forms differ quantitatively in their antibacterial efficiency both *in vitro* and *in vivo*.[1] Furthermore penicillin esters have been described which, although inactive *in vitro*, are highly effective *in vivo* in certain species of animals.[9] The residual form of streptomycin and crystalline streptomycin also differ quantitatively *in vitro*. The residual form is a more efficient antibacterial agent against the typhoid bacillus *in vitro*, and possesses definite *in vivo* activity as well. Its value as a chemotherapeutic agent, nevertheless, must await further pharmacological and therapeutic tests.

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[8] Fried, J., and Titus, E., *J. Biol. Chem.*, 1947, **168**, 391.

Summary. The sensitivity of *E. typhosa* to a residual fraction of streptomycin obtained during the purification process is at least 2 to 5 times greater than to the crystalline $CaCl_2$ double salt of streptomycin. By the Oxford cup plate method, the *E. typhosa-Bc. subtilis* (or *E. coli*) differential ratio of this material is approximately 2.0-3.0. This residual streptomycin is active *in vivo* as well as *in vitro*. Its activity *in vivo* is not as great, however, as might be anticipated from the *in vitro* results.

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[9] Meyer, K., Hobby, G. L., and Dawson, M. H., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 100.

15925

Complement Fixation Studies with Pus Antigen in Granuloma Inguinale.

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Three types of antigens for use in complement fixation tests for granuloma inguinale have been described. Anderson and her associates[1] utilized "capsular substances" obtained by chemical treatment of embryonic yolk cultures of *"Donovania granulomatis."* Dunham and Rake[2] grew a strain obtained from Anderson on yolk-beef heart infusion agar and then on Levinthal's medium, from which they prepared culture antigens. Jennison *et al.*[3] used saline suspensions of Donovan bodies grown in the yolks of fertile eggs.

This report presents data from use of an antigen prepared from pus aspirated from an abscess of Donovan body origin. This abscess was one of 3 which followed an initial lesion of the cervix uteri. The diagnosis of granuloma inguinale of the cervix was established by smear and biopsy.[4]

Serological Studies. The pus antigen, used in complement fixation tests for granuloma inguinale, was prepared from pus aspirated from a large fluctuating abscess of the hand. Smears revealed typical Donovan bodies in abundance. No other organism could be demonstrated. The pus was diluted 1:6 with physiological saline solution, shaken thoroughly, and heated at 60°C for 1 hour on 2 successive days. Merthiolate (1:10,000) was added. All tests were negative for organisms which might be expected to grow on the media employed.

The pus antigen was used in complement fixation tests with sera from 25 patients with proven granuloma inguinale, from 14 hospitalized individuals with various infections, other than venereal, from 12 syphilitic patients with no evidence of granuloma inguinale, and from 5 healthy individuals who served as controls.

As shown in Table I, 21 (84%) of the

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[1] Anderson, Katherine, Goodpasture, E. W., and De Monbreun, W. A., *J. Exp. Med.*, 1945, **81**, 41.

[2] Dunham, Wolcott, and Rake, Geoffrey, *J. Bact.*, 1946, **51**, 67.

[3] Jennison, David B., Helwig, Elson B., and Milstone, J. H., *Arch. Dermat. and Syph.*, 1947, **55**, 342.

[4] Packer, Henry, Turner, Henry B., and Dulaney, Anna Dean, *J. A. M. A.*, in press.

TABLE I.
Results of Complement Fixation Tests with Antigen Prepared from Pus Containing Donovan Bodies.

Clinical categories		No. of patients	C.F.	
			Positive	Negative
Clinical granuloma inguinale, verified by positive smears	Duration of lesions, 6 mo. or longer, with one exception	25	21	4*
No evidence of clinical granuloma inguinale	Hospital patients, various clinical categories	14	0	14
	Syphilitic patients with high Wassermann titers	12	4	8
	Healthy individuals, no evidence of disease	5	0	5

* This group included one patient with a very early discrete lesion, and one whose lesion had been healed for some time.

25 patients with granuloma inguinale gave positive complement fixation tests with the Donovan body antigen. Nineteen of the 21 sera were strongly positive and gave reactions of 3-4+ in dilutions of 1:5 and 1:10. Four sera were positive in a dilution of 1:20 with an antigen dilution of 1:20. One patient with a very early lesion, which was small and discrete, gave a negative reaction in the serum dilution of 1:5. Another patient whose lesions had apparently healed also gave a negative reaction. The highest antibody titers were demonstrated in patients who had been treated but who showed reactivity of lesions after a period of latency. Remissions after treatment and apparent healing occur frequently in this disease.

All of the 14 hospital patients with various types of clinical findings, other than granuloma inguinale, gave negative reactions, as did the 5 healthy individuals who served as controls. Four of the 12 syphilitic patients gave positive reactions with the pus antigen. These patients were under treatment for neurosyphilis, and quantitative Wassermann tests revealed high serum titers. Since one venereal disease (syphilis) was present, the possibility of multiple infection must be recognized even though no history or clinical evidence of granuloma inguinale could be elicited. The paucity of pus antigen prevented more extensive control studies.

Coincident complement fixations for lymphogranuloma venereum and granuloma venereum were carried out with the sera of 13 patients with proven granuloma inguinale and of 6 patients with syphilis. Six of the 13 patients with established granuloma inguinale gave positive reactions for lymphogranuloma venereum in significant serum dilutions. The 7 other sera of this group gave negative reactions, as did the 6 syphilitic sera. These 6 positive reactions for lymphogranuloma venereum were accepted as evidence of multiple infection rather than cross reactivity, on the basis of serum titers. It has been pointed out[5] that a serum titer of 1:40 offers good evidence of infection with the virus of lymphogranuloma venereum. It should be emphasized that the patient from whom the pus for antigen was obtained was serologically negative for this disease and that the Frei test was also negative.

If antigens were available, serological study of granuloma inguinale would undoubtedly afford valuable information. Obviously, cultures of the causative organism would be expected to offer the most dependable source of material for such antigens.

While pus sources of antigens, as used by us, are not practical because of the rare occasions when such material would be available, the sensitivity of this preparation was apparent. Anderson *et al.*[3] found that use of undiluted patient's serum and a 1:10 dilution of the "capsular" antigen gave the best results in complement fixation tests. Direct comparison is impossible because of the differences in antigens and technics, but it would appear that tests carried out in this laboratory using serum dilutions of 1:5 to 1:20 and antigen dilutions of 1:10 (1:60 dilution of pus) and 1:20 (1:120 dilution of pus) gave even more sensitive antigen-antibody reactions.

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[5] Dulaney, Anna Dean, and Packer, Henry, *J. Immunol.*, 1947, **55**, 53.

Additional evidence of the sensitivity of the pus antigen is offered by the following incident. A blood specimen, sent to our laboratory, carried the request for a complement fixation test for "granuloma inguinale." Even though it was believed that a mistake had been made and that the intention was to request an examination for "lymphogranuloma inguinale" (venereum), the serum was tested with the Donovan body antigen. The serum gave a 4+ reaction in a dilution of 1:20. Inquiry revealed that the patient had a perianal ulcerative lesion. A request was made for smears from this lesion, and abundant Donovan bodies were demonstrated. In this instance the patient gave a typical clinical picture of lymphogranuloma venereum with bilateral inguinal buboes and a positive Frei test; the granuloma inguinale had not been recognized.

Conclusion. An antigen prepared from pus aspirated from a metastatic abscess containing Donovan bodies yielded highly sensitive and specific serological evidence of granuloma inguinale when used in complement fixation tests. Twenty-one (84%) of 25 patients with established granuloma inguinale (positive smears) gave positive reactions.

15926 P

Enhancement of Diabetes Produced by Adrenocorticotropic Hormone in Rats Maintained on a Carbohydrate Free Diet.*

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Introduction and Methods. It has been reported by Ingle, Li and Evans[1] that pure adrenocorticotropic hormone produces glycosuria and increases nitrogen excretion in normal rats forced fed a high carbohydrate diet. Subsequent work from this laboratory has shown that adrenocorticotropic hormone enhances the glycosuria and nitrogen excretion of diabetic rats maintained on a diet containing approximately 52% preformed carbohydrate. It should be noted that the amount of glucose in the urine in the above experiments was less than the ingested preformed carbohydrate, so that gluconeogenesis did not have to be invoked to account for the glycosuria. It therefore seemed that a more severe test of the diabetes-enhancing activity of adrenocorticotropic hormone would be furnished by a study of its metabolic effects in diabetic rats maintained on a carbohydrate-free diet. Under such conditions glycosuria could only be accounted for by gluconeogenesis.

In these experiments rats with alloxan-diabetes were used and the methods and

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* Supported by grants from the James Foundation of the Medical School and the Research Board of the University of California.

[1] Ingle, Dwight J., Choh Hao Li, and Evans, Herbert M., *Endocrinology*, 1946, **39**, 32.

[2] Bennett, Leslie L., and Choh Hao Li, in press.

technics were those previously described,[2] the sole exception being that the diet consisted of casein 70½%, crisco 24%, salts 4%, water soluble vitamin mixture 1%, and liver powder ½%. Fat soluble vitamins were administered once a week and a constant amount of diet was fed daily throughout the whole experimental period. Three mg of adrenocorticotropic hormone was administered per day.

Results. In Table I are presented the data from the experiment. It will be noted that all animals exhibited a pronounced glycosuria prior to injection and that the D/N ratios varied from 0.99 to 3.43. It also is apparent that in all experiments the mean glycosuria and nitrogen excretion was greater during the hormone injection period than during either the pre- or the postinjection control periods. In 4 of the 5 experiments the increases in glycosuria and nitrogen excretion were statistically significant. In the one case, rat B5044, in which the change was not significant, the initial severity of the diabetes was almost maximal as judged by the degree of glycosuria and the D/N ratio of 3.43. All of the animals lost weight during the hormone treatment period while they all gained during at least one of the control periods.

Comment. Although conclusions based upon D/N ratios are open to doubt it may be pointed out that the classical interpretation of the D/N ratios in these experiments would not require the assumption of gluconeogenesis from fat since the D/N ratios never exceeded 3.65. The increase in protein catabolism as reflected by the rise in urinary nitrogen was inadequate to account for the extra urinary glucose as having been derived solely from this additional protein being broken down. These observations would imply that not only is adrenocorticotropic hormone concerned with carbohydrate metabolism by increasing gluconeogenesis from protein but also by depressing carbohydrate utilization.

Summary. Pure adrenocorticotropic hormone enhances both the glycosuria and the nitrogen excretion of diabetic rats maintained on a carbohydrate-free diet.

TABLE I.
Effect of 3 mg of Adrenocorticotropic Hormone per Day on Daily Glycosuria and Nitrogen Excretion of Diabetic Rats Maintained on a Carbohydrate Free Diet.

		First control period				Hormone injection period				Second control period			
Rat No.	G of diet per day	Mg urinary glucose per day	Mg urinary N. per day	D/N	Gain or loss of wt	Mg urinary glucose per day	Mg urinary N. per day	D/N	Gain or loss of wt	Mg urinary glucose per day	Mg urinary N. per day	D/N	Gain or loss of wt
G5439	10	1150±170* (12)†	678±15	1.70	+	1810±130 (5)	742±13	2.44	—	400± 90 (5)	634±31	.63	+
p‡		.02	.02							<.01	<.01		
B5044	10	3090± 70 (12)	901±10	3.43	—	3170±130 (5)	929±20	3.42	—	2840±140 (5)	881±25	3.23	+
p		.50	.20							.10	.10		
G5031	8	2060± 70 (15)	715± 6	2.88	—	2420±120 (5)	769±15	3.15	—	1640± 70 (5)	604±24	2.82	+
p		.02	<.01							<.01	<.01		
W5037	8	710± 90 (12)	718± 9	.99	0	1650±260 (5)	771±23	2.14	—	280± 80 (5)	673±25	.42	+
p		<.01	.015							<.01	.01		
W5090	12	2530± 70 (15)	1010± 9	2.51	+	3210±260 (5)	1090±20	2.94	—	3090± 70 (5)	1037±13	2.98	+
p		<.01	<.01							.60	.07		

* Standard deviation.
† Number of days observation.
‡ From Fisher's table of t.

15927 P

Effect of Pentaquine on Cardiovascular System of Unanesthetized Dogs.

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In the course of studies on the chemotherapy of malaria with pentaquine, Craige, Jones, Eichelberger, Alving, Pulman, and Wharton[1] observed that a certain number of patients developed postural hypertension which they believe was caused by the drug. More recently, Freis and Wilkins[2] have summarized the results of a clinical trial of this compound in a group of 17 patients with long standing hypertension. In the majority of their cases the administration of large doses produced lowering of the systolic and diastolic pressure in the supine position, usually associated with postural hypotension. Pentaquine is too toxic for routine treatment of hypertension in man, but it is unknown at the present time whether it might not be possible to obtain less toxic, and more active agents in the group of 8-amino quinolines. The search for such an agent would be made easier if it were possible to use an experimental animal as a test object. For this reason we have investigated the effects of pentaquine on the cardiovascular system of normal unanesthetized dogs, and have obtained results suggesting that such studies can serve as the basis for a screening test for other 8-amino quinolines which may have an action on essential hypertension in man.

Unanesthetized adult dogs were used throughout. Animals were comfortably restrained on a tilt table during the period of observation. Arterial pressure was recorded by an Anderson glass capsule manometer[3] from a cannula inserted in a femoral artery. One per cent procaine was used to anesthetize the skin over the area of the incision.

The response of the cardiovascular system to the following procedures was studied: (a) The effect of the intravenous injection of a range of doses of epinephrine, (b) the effect of the intravenous injection of a range of doses of acetylcholine, (c) the effect of tilting from a horizontal position to an 85° angle, for a period of one minute.

Following control observations the cannulated artery was tied, powdered sulfadiazine shaken into the incision, and the wound closed with linen sutures. Pentaquine* was then administered orally as an aqueous solution 3 times daily at 8 a. m., 12 m. and 5 p. m. After an appropriate period of treatment the observations on the cardiovascular system were repeated, using the opposite femoral artery.

Results. Typical toxic effects were produced in all dogs by the oral administration of doses of pentaquine in excess of 3.75 mg

TABLE I.
Resting Observations on Unanesthetized Dog Before and After Treatment with Pentaquine.

Dose mg/kg	Length of treatment	No. of dogs	Dog showing postural hypotension	Mean pulse rate per min.	Mean arterial pressure mm Hg	
					Diastolic	Systolic
—	—	12	0/12	119	119	171
3.75	4½	6	0/6	135	125	195
7.5	4½	5	2/5	151	103	146
15.0	2½	6	4/6	139	108	148
30.0	2½	5	4/5	131	95	127

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[1] Craige, Jones, Eichelberger, Alving, Pulman, and Wharton, personal communication.

[2] Freis and Wilkins, personal communication.

[3] Anderson, F., *J. Lab. Clin. Med.*, 1941, **26**, 1520.

* All doses of pentaquine used refer to the monophosphate salt.

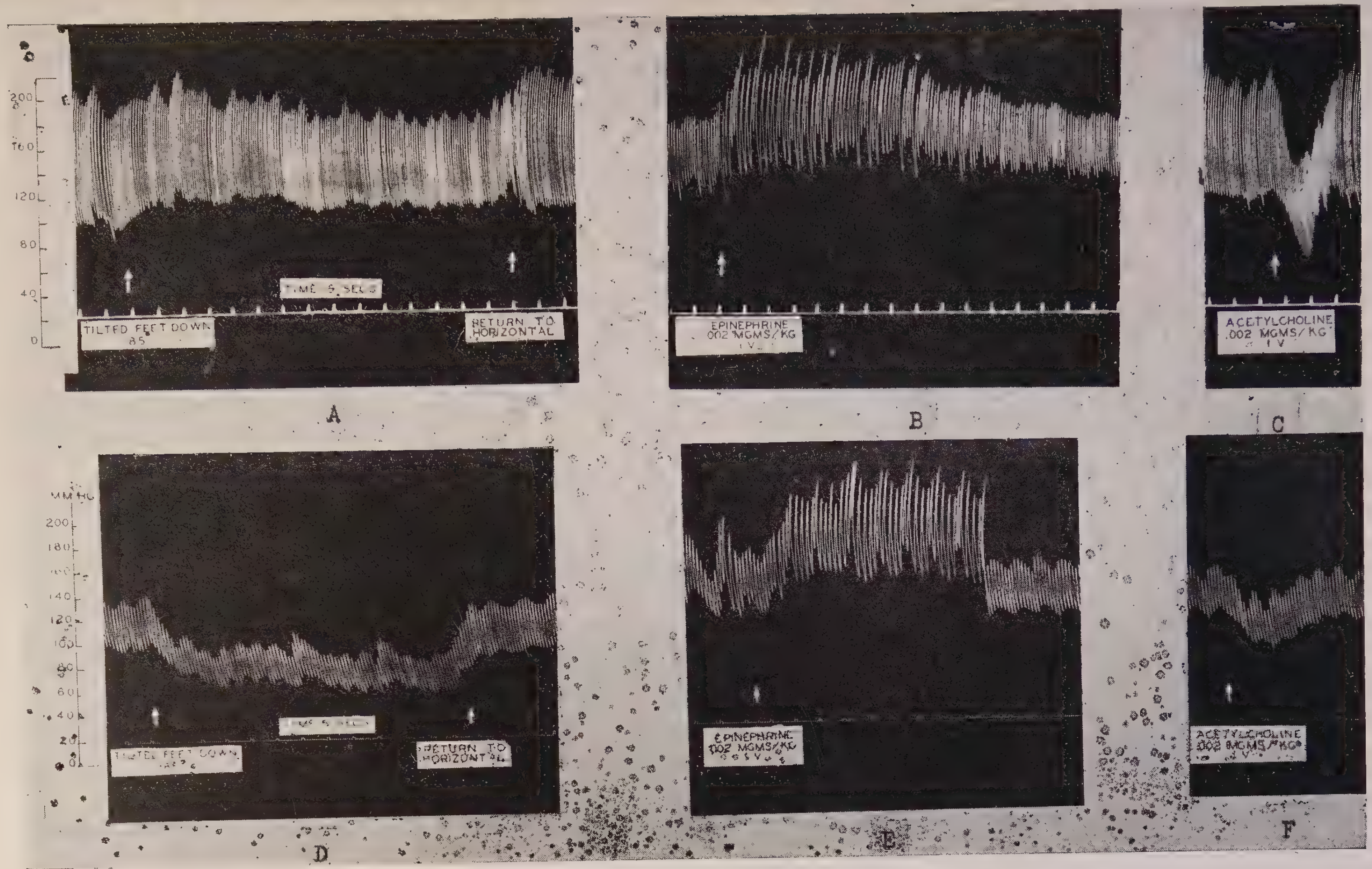

Fig. 1. The effect of pentaquine on response of the cardiovascular system to tilting, epinephrine, and acetylcholine. Fig. A, B, C, responses to an untreated unanesthetized dog. Fig. D, E. F, responses after treatment with pentaquine, 15 mg per kilo per day for 4½ days.

TABLE II.
Effect of Pentaquine on Cardiovascular Responses of Unanesthetized Dogs to Tilting, Epinephrine and Acetylcholine.

		Mean % change from resting observations.								
		Tilting feet down 85° for one minute			I.V. epinephrine 0.002 mg per kilo			I.V. acetylcholine 0.002 mg per kilo		
			Arterial pressure			Arterial pressure			Arterial pressure	
	No. dogs	Pulse rate	Systolic	Diastolic	Pulse rate	Systolic	Diastolic	Pulse rate	Systolic	Diastolic
Untreated controls	12	+16.6	+ 3.1	+ 8.9	—30.8	+31.2	+28.5	+48.0	— 26.6	—31.1
Pentaquine 3.75 mg/K/day	6	+ 8.2	— 8.6	— 3.0	—36.5	+36.7	+35.7	+55.5	—29.4	—28.5
,, 7.5 ,, ,, ,,	5	+19.5	—18.1	— 7.3	—43.6	+36.2	+24.9	+25.1	—19.3	—18.2
,, 15.0 ,, ,, ,,	6	+23.6	—25.5	—25.8	—43.8	+42.1	+38.1	+21.1	—24.3	—27.8
,, 30.0 ,, ,, ,,	5	+ 8.9	—29.8	—29.8	—36.2	+48.1	+48.1	+20.1	—19.6	—19.4

per kg per day. These consisted of methemoglobinemia, loss of appetite, and generalized depression. In addition, most animals showed constricted pupils, some endophthalmus, and relaxation of the nictitating membrane.

Table I summarizes observations on the pulse and blood pressure of treated and untreated dogs lying quietly on the tilt table. In general the pulse rate following treatment with pentaquine was more rapid, and both diastolic and systolic arterial pressures were lower than in the control animals. The toxicity of doses of 30 mg per kg per day was such that treatment was limited to a period of 2½ days. On doses of 3.75 mg per kg per day, animals survived at least 2 weeks medication.

Fig. 1 illustrates a representative experiment. Kymograph records, 1-A, B, and C show the response of the circulation of an untreated animal to tilting, epinephrine, and acetylcholine. Kymograph records D, E, and F of Fig. 1 show the response of the circulation, following treatment with pentaquine for 4½ days at a total daily dose of 15 mg per kg.

The most striking effect of pentaquine was the development of postural hypotension, unaccompanied by significant tachycardia. Also characteristic of those animals showing postural hypotension was the failure of acetylcholine to accelerate the pulse.

Table II summarizes the data relating dosage of pentaquine to effect on the cardiovascular system. Insignificant changes were produced by daily doses of 3.75 mg per kg for 4½ days. Doses of 7.5 mg per kg per day produced postural hypotension in part of the animals, and higher doses produced a typical effect in most of the animals.

An important feature of the action of pentaquine was the length of time required for production of the cardiovascular effects. In 3 dogs the repeated intravenous injection of 1 mg per kg of pentaquine was fatal when 10-12 mg per kg had been administered over a period of one hour. Death in such animals was accompanied by generalized convulsions ending in respiratory failure, without evidence of methemoglobin. In none of the 3 dogs thus studied was there a significant change in cardiovascular response to tilting, epinephrine, or acetylcholine. In other animals repeated sublethal doses of pentaquine did not lead to postural hypotension until at least 2½ days of treatment had elapsed.

None of the 6 animals given daily doses of 3.75 mg per kg of pentaquine for 4½ days showed a significant postural hypotension. Treatment of this group of dogs was continued for 4 days longer at which time one of the 6 showed a fall in blood pressure on tilting. It would appear from this that maximal cardiovascular effects from a particular dose might ordinarily be expected by 5 days, if they are to occur at all.

Also characteristic of these cardiovascular actions of pentaquine was a persistence of effect beyond the period of therapy. In some animals postural hypotension was observed for at least 5 days after drug administration had been discontinued. These same phenomena of slow onset, and persistence of action have been observed in man.[1,2]

Discussion. A consideration of the results presented above permits the following provisional analysis: (a) Since epinephrine in pentaquine-treated animals produces at least as great a degree of hypertension as in controls, one must assume that the peripheral sympathetic end organs are intact, (b) since epinephrine produces as much bradycardia the so-called "vagus cut off" mechanism is intact, (c) since acetylcholine still produces a fall in blood pressure the peripheral end organs of the parasympathetic system are unaffected, (d) the inability of the pulse to speed up with a fall in blood pressure either as a result of tilting or acetylcholine suggests that reflexes necessary for maintaining arterial pressure have been altered. These findings therefore taken in conjunction with those of Moe and Seevers[4] suggest that the cardiovascular actions of 8-amino quinolines are produced by a functional impairment of the central sympathetic system.

Conclusions. The repeated administration of high doses of pentaquine produces an effect upon the cardiovascular system such that unanesthetized animals develop a striking postural hypotension. Pentaquine has no effect upon the response of animals to epinephrine, but blocks the tachycardia produced by acetylcholine. It is suggested that these effects are produced by an impairment of the central portion of the sympathetic nervous system.

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[4] Moe, G. K., and Seevers, M. H., *Fed. Proc.*, 1946, **5**, 193.

15928

The Circulatory Action of a Number of Phenylpropylamine Derivatives.*

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Barger and Dale[1] reported that phenethylamine presented the optimal basic chemical structure for sympathomimetic activity. This has been adequately confirmed by the synthesis and pharmacological examination of numerous modifications of the phenethylamine skeleton. A number of derivatives (Table I) in which an ethylamine side chain is separated from the benzene ring by either a keto or hydroxyl group have been made

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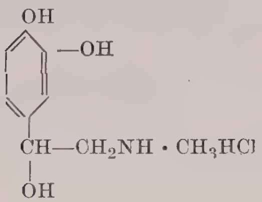
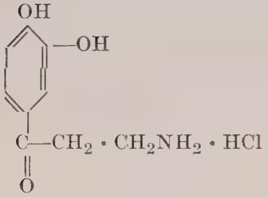
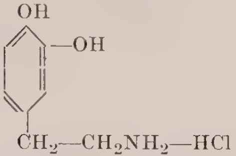
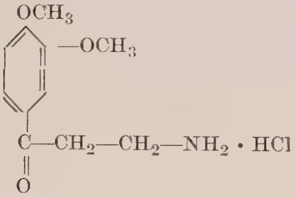
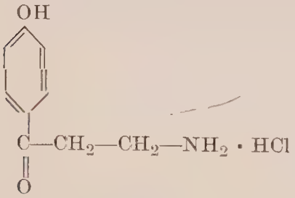
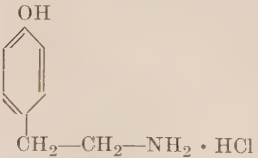
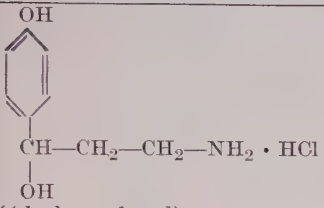
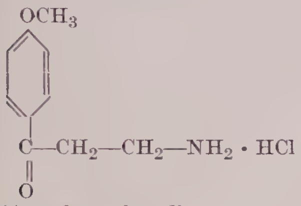
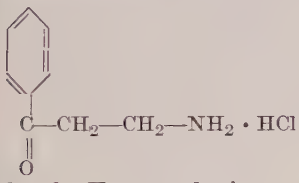
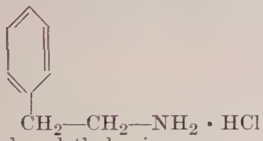
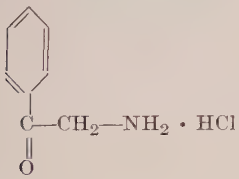
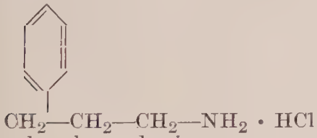
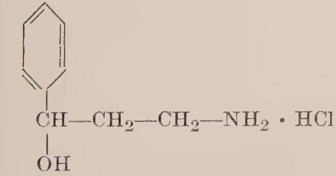
* This investigation was supported in part by the Smith, Kline and French Laboratories Fellowship Fund.

[1] Barger, G., and Dale, H. H., *J. Physiol.*, 1910, **41**, 19.

TABLE I.
Comparative Pressor Activity.

Formula	Code	Dose ranges compared		Pressor equivalent (Epinephrine = 1)
		mg/kg	millimol/kg	
 Epinephrine	Epin.	0.0025 to 0.005	0.0000115 to 0.000023	1
 γ-(3,4-dihydroxyphenyl)-γ-Ketopropylamine	di-OH-K-Pr. (I)	0.5 to 1.0	0.0023 to 0.0045	1/100-1/200
 β-(3,4-dihydroxyphenyl) ethylamine	di-OH-Et. (II)	0.15 to 0.2	0.0008 to 0.0011	1/35-1/50
 γ-(3,4-dimethoxyphenyl)-γ-Ketopropylamine	di-me-K-Pr. (III)	10.0 to 12.5	0.040 to 0.051	<1/5000
 γ-(4-hydroxyphenyl)-γ-Ketopropylamine	OH-K-Pr. (IV)	1.5 to 3.0	0.0075 to 0.015	1/350-1/700
 β-(4-hydroxyphenyl) ethylamine	OH-Et. (V)	0.2 to 0.4	0.00115 to 0.0023	1/50-1/100

Formula	Code	Dose ranges compared		Pressor equivalent (Epinephrine = 1)
		mg/kg	millimol/kg	
 γ-(4-hydroxyphenyl)-γ-hydroxypropylamine	OH-Pr.-OH (VI)	1.5 to 3.0	0.0073 to 0.015	1/350-1/700
 γ-(4-methoxyphenyl)-γ-Ketopropylamine	Mc-K-Pr. (VII)	10.0 to 12.5	0.043 to 0.054	<1/5000
 γ-phenyl-γ-Ketopropylamine	K-Pr. (VIII)	2.5 to 5.0	0.0135 to 0.027	1/1200-1/2400
 β-phenylethylamine	Et. (IX)	0.15 to 0.3	0.00095 to 0.0019	1/100-1/200
 β-phenyl-β-Ketoethylamine	K-Et. (X)	0.4 to 1.0	0.00235 to 0.00587	1/200-1/500
 γ-phenylpropylamine	N-Pr. (XI)	2.0 to 4.0	0.0115 to 0.0233	1/1000-1/2000
 γ-phenyl-γ-hydroxypropylamine	Pr.-OH (XII)	4.0 to 8.0	0.0213 to 0.043	<1/4000

available† to us. It appeared desirable to evaluate these agents from the standpoint of their relationship to the pressor amine field. In the present experiments, atropinized dogs anesthetized with pentobarbital sodium received intravenous injections of small doses of epinephrine until a constant response was obtained. The animals then were standardized by injecting a wide range of doses of epinephrine. After standardization, a dose of one of the new compounds was injected intravenously, the response obtained compared with that of an equi-effective standard dose of epinephrine, and the epinephrine equivalent subsequently calculated. In other experiments the epinephrine equivalents of well-characterized phenethylamine‡ and phenylpropylamine‡ compounds were determined in the manner described for the new propylamine derivatives. In order to obviate the complicating factor of tachyphylaxis, only one dose of a compound was injected in a given animal in all of the present comparative pressor activity experiments.

Di-Substituted Phenyl Derivatives. The rise of blood pressure produced by 0.000023 millimol per kg of body weight§ (0.005 mg per kg of body weight§) of epinephrine (Fig. 1, A) was approximated in intensity by di-OH-K-Pr. (Fig. 1, B) in a dose range of 0.0023-0.0045 millimol (0.5-1.0 mg); however, duration of pressor action was more prolonged in the case of the propylamine derivative. Pressor activity comparable with the above doses of di-OH-K-Pr. and epinephrine was obtained following 0.0008-0.0011 millimol (0.15-0.2 mg) di-OH-Et (Fig. 1, C). Methylation of both hydroxyls of di-OH-K-Pr. results in a compound, di-me-K-Pr., which produced an initial depressor followed by a negligible pressor effect (Fig. 1, D).

Mono-Substituted Phenyl Derivatives. Pressor responses were obtained with 0.075-0.015 millimol (1.5-3.0 mg) of OH-k-Pr. (Fig. 2, A) which were comparable in intensity with those obtained following 0.00115 millimol (0.2 mg) of OH-Et. (Fig. 2, B); however, duration of pressor action following injection of the propylamine compound always was more prolonged than that obtained after the ethylamine derivative. A rise in blood pressure was caused by 0.0073-0.015 millimol (1.5-3.0 mg) OH-Pr.-OH (Fig. 2, C) which in intensity approximated that observed after the above doses of the propyl- and ethylamine compounds. Me-K-Pr. (Fig. 2, D) in doses of 0.054-0.043 millimol (12.5-10.0 mg) produced an initial depressor followed by a slight pressor effect.

Non-Substituted Phenyl Derivatives. The intensity of pressor action following 0.0135-0.027 millimol (2.5-5.0 mg) K-Pr. (Fig. 3, A) was of the same order as that obtained after 0.0019 millimol (0.3 mg) Et. (Fig. 3, B), as well as that observed on injection of 0.00587 millimol (1.0 mg) of K-Et. (Fig. 3, C) or after 0.01725 millimol (3.0 mg) of n-Pr. (Fig. 3, D). A negligible pressor effect was produced after 0.0216-0.043 millimol (4.0-8.0 mg) Pr.-OH (Fig. 3, E).

Discussion. From the standpoint of intensity of pressor action, di-hydroxy-γ-keto-phenylpropylamine (I) was the most active of the new derivatives (Table I). Compound I exhibited 1/100-1/200 the pressor activity of epinephrine and 1/5 to 1/3 the activity of dihydroxy-phenethylamine (II) (Table I). Dimethoxy-γ-ketophenylpropylamine (III) evidenced less than 1/5000 the pressor activity of epinephrine (Table I). Hydroxy-γ-ketopropylamine (IV) was 1/8 as active as hydroxy-phenethylamine (II) and 1/350 to 1/700 as active as epinephrine (Table I). Hydroxy-phenyl-n-propanolamine (VI) manifested the same degree of pressor activity as that of its keto-analogue (Table I). Mono-

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† Compounds I, III, IV, VI, VII, VIII, XII (Table I) were prepared and made available to us by the late Garfield Powell, Professor of Organic Chemistry, Columbia University. Compound VIII was prepared previously by Mannich, C., and Abdullah, S. M. (Ber. 68B: 113-20, 1935), but has not been investigated pharmacologically. All of the other above agents are new derivatives.

‡ We are indebted to Dr. Gordon A. Alles for samples of compounds II, V, IX, X, and XI (Table I).

§ Throughout the remainder of the text it is understood that all doses mentioned are per kilogram of body weight.

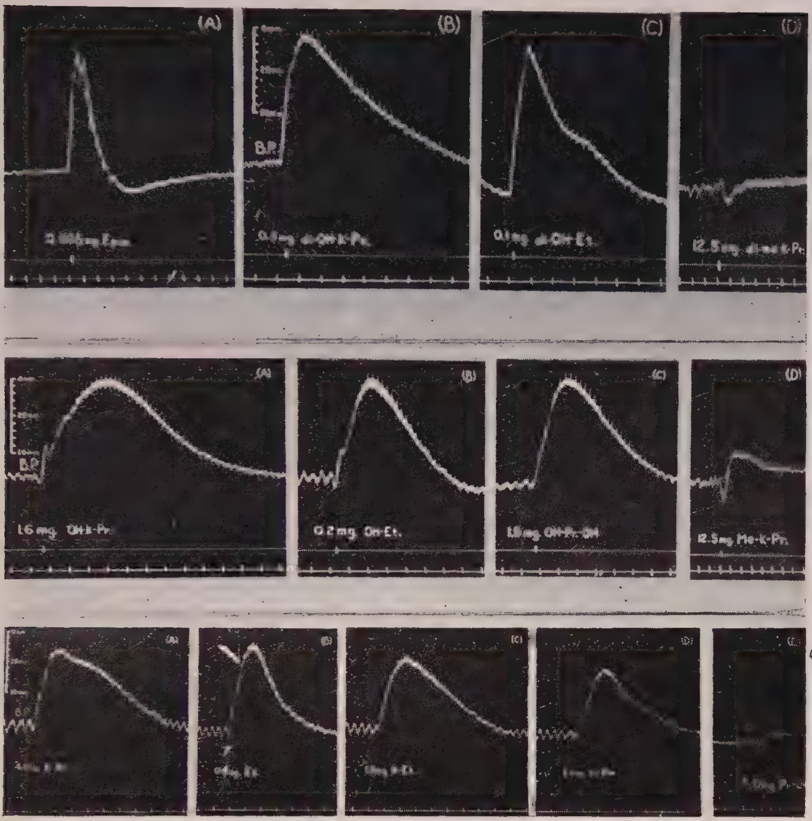

FIG. 1, top, FIG. 2, middle, FIG. 3, bottom. Atropinized (2.0 mg/kg) dogs anesthetized with pentobarbital sodium. B.P.—carotid blood pressure by mercury manometer. Upper lines indicate injection into a femoral vein. Lower lines indicate time in minutes. Doses are in mg per kilogram of body weight.

methoxy-γ-ketophenylpropylamine evidenced a low order of pressor effectiveness, being <1/5000 as active as epinephrine. γ-ketophenylpropylamine (VIII) possessed 1/16 the activity of phenethylamine (IX) and 1/1200 to 1/2400 the activity of epinephrine (Table I). The effectiveness of γ-ketophenylpropylamine (VIII) was slightly less than that of phenyl-n-propylamine (XI). Phenyl-n-propanolamine (XII) exhibited less than 1/4000 the pressor activity of epinephrine and was therefore much less active than its corresponding keto-derivative. The interrelationship between aryl and alkylamine substitution is, therefore, apparent because derivatives IV and VI were equi-active pressor agents.

Summary. The intravenous circulatory activity of the following phenylpropylamines was compared with that of corresponding phenethylamine derivatives and epinephrine in atropinized dogs anesthetized with pentobarbital sodium: γ-(3,4-dihydroxyphenyl)-γ-Ketopropylamine (I); γ-(4-hydroxyphenyl)-γ-Ketopropylamine (IV); γ-(4-hydroxyphenyl)-γ-hydroxypropylamine (VI); γ-phenyl-γ-ketopropylamine (VIII); γ-phenyl-γ-hydoxypropylamine (XII); γ-(4-methoxyphenyl)-γ-Ketopropylamine (VII); γ-(3,4-dimethoxyphenyl)-γ-Ketopropylamine (III). These derivatives all were found to exhibit some degree of pressor activity. The above listing is in the order of their decreasing pressor effectiveness. The most active compound (I) manifested 1/100-1/200 and the least active agent (III) possessed <1/5000 the activity of epinephrine.

15929

Do the Human Ventricles Eject Simultaneously?*

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and A. C. Witham.

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The lack of an accurate measure of the transmission time of the pulse from the left ventricle to the carotid artery has been a critical deficiency in a study of the forces which give rise to the deflections of the ballistocardiogram. For the I-wave on the ballistocardiogram which, from all theoretical constructions, should mark the beginning of the cardiac ejection, does not start, on the average, until 5 msec. before the upstroke of a carotid surface pulse.[1] This short interval would indicate that the ballistocardiograph is insensitive to the first part of the ejection. How large a part depends upon whether the estimate of 60 msec. for the transmission time from apex beat to subclavian artery,[2] or the estimate of 30 msec. from heart to carotid, based on stretch measurements made upon isolated aortic rings,[1] is nearer the correct figure.

In a series of patients in whom the right ventricles were catheterized, we have recorded simultaneously the right ventricular pressure, carotid surface pulse and ballistocardiogram, to determine directly this transmission interval from heart to carotid. Unexpectedly, the data do not give evidence on this point, for they indicate that the ejection does not start from the 2 ventricles simultaneously.

Method. A silk X-ray opaque catheter was passed through an antecubital vein into the right ventricle, using the technic of Cournand *et al.*[3] Whenever possible, the catheter was also passed into the pulmonary artery to record pulmonary diastolic pressure levels. The catheter was connected to an optical manometer of adequate frequency for pressure recording. The carotid surface pulse was recorded by funnel connected, with air transmission, to a rubber diaphragm bearing a mirror. The ballistocardiograph was of the high frequency type. All recording light beams were passed into the same camera, and all were cut by a single timer clock, so that parallax was measurable on the records themselves.

For an estimate of the time lags inherent in the recording systems, a rubber bag filled with air was placed on the ballistocardiograph. The catheter was passed through a stopper into the bag to record pressure change. The funnel connected to the surface pulse recorder was placed on the surface of the bag, and rapid pressure oscillations within the bag were generated by gentle taps. In a series of 10 trials, the interval between the start of the ballistocardiographic recoil and the start of the pressure change as recorded by catheter was 0.8 msec., and that between ballistocardiogram and surface pulse recorded 3.6 msec.

Results. The timing of the records taken from 10 individuals are given in Table I, with an example in Fig. 1. All events were dated from the beginning of ejection in the right ventricle, as determined by the point on the ventricular pressure rise when the pulmonary diastolic pressure level was reached. In 2 cases (marked by asterisk), pulmonary pressure tracings were not available, and the beginning of ejection was placed by inspection. In only 2 of the 10 cases is the time interval reasonable for pulse transmission from heart to carotid. In 4, the surface pulse upstroke actually precedes the beginning of ejection. Since we have no evidence that

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* This investigation was aided by a grant from the Life Insurance Medical Research Fund.

[1] Hamilton, W. F., Dow, P., and Remington, J. W., *Am. J. Physiol.*, 1945, **144**, 557.

[2] Bazett, H. C., Cotton, F. S., LaPlace, L. B., and Scott, J. C., *Am. J. Physiol.*, 1935, **113**, 312.

[3] Cournand, A., Ranges, H. A., and Riley, R. L., *J. Clin. Invest.*, 1942, **21**, 287.

TABLE I.
Timing of Events from Start of Right Ventricle Ejection.

				Ballistocardiogram	
Subject	Symptomology	Brachial pressure, mm Hg	Start of carotid surface pulse, msec.	Start of H wave msec.	Start of I wave, msec.
1	Congestive failure	146/54	— 5		
2*	Malignant hypertension	290/190	+30	—40	+10
3*	Compensated congestive failure	138/75	—25	—75	—25
4	Congestive failure	128/70	+10	—70	— 5
5	” ”	125/80	+45	?	+ 5
6	” ”	101/73	0	—30	+ 5
7	Normal	110/80	—50	—50	—30
8	”	90/60	+ 5	—40	— 5
9	”	140/100	+15	—30	+10
10	”	152/100	—10	—25	0
Mean			+ 1	—45	— 4

an artefact in the recording can account for this discrepancy, we are faced with the conclusion that the left ventricle begins its ejection earlier than the right. That asynchronism of the beginning of isometric contraction may be present in the exposed dog heart was pointed out by Katz.[4] In his records, either ventricle might precede, and the average time difference was of the order of 13 msec. The time differences recorded here are considerably larger than this.

The human tracings of Bloomfield *et al.*[5] indicate a lag of the order of 80 msec. from the first part of the R wave of the electrocardiogram, to the beginning of the pressure rise in the right ventricle, whereas the ventricular contraction should start during the last part of the R wave.[6] This is seemingly further evidence in favor of an asynchronization of the 2 ventricles.

The start of the I-wave of the ballistocardiogram does not differ greatly in time from the upstroke of the carotid pulse, as noted before.[1] In fact, the start of the H-wave seems roughly related to the start of left ventricle ejection, and the I-wave usually does not develop until right heart outflow has begun.

That the right ventricle may play a larger role in determining the initial deflections of the ballistocardiogram than any proposed theoretical construction of the forces would allow is suggested by the recent evidence of Starr and Friedland.[7] They found that in inspiration, the ballistocardiographic recoil was increased, and in expiration, decreased, as might be expected if the forces generated were related to changes in right heart output as opposed to left heart output. Our records show the same changes they have reported. In addition, however, we[8] find that in a straining with closed glottis (Valsalva's experiment), the ballistocardiographic recoil is also increased, which seemingly cannot be correlated with output changes from either ventricle.

Summary. On the basis of simultaneously recorded right ventricular pressures, carotid surface pulses and ballistocardiographic recoils, evidence is presented that ejection from the right and left ventricles may not start simultaneously in the human. The I-wave of the ballistocardiographic record seemingly dates to the right heart ejection, and is not initiated by left ventricle ejection which must have started earlier.

Since this paper was submitted, an abstract appeared in the *Federation Proceed-*

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[4] Katz, L. N., *Am. J. Physiol.*, 1925, **72**, 655.

[5] Bloomfield, R. A., Lauson, H. D., Cournand, A., Breed, E. S., and Richards, D. W., Jr., *J. Clin. Invest.*, 1946, **25**, 639.

[6] Wiggers, C. F., *Circulation in Health and Disease*, 267, Lea and Febiger, Philadelphia, 1923.

[7] Starr, I., and Friedland, C. K., *J. Clin. Invest.*, 1946, **25**, 53.

[8] Hamilton, W. F., and Dow, P., unpublished observations.

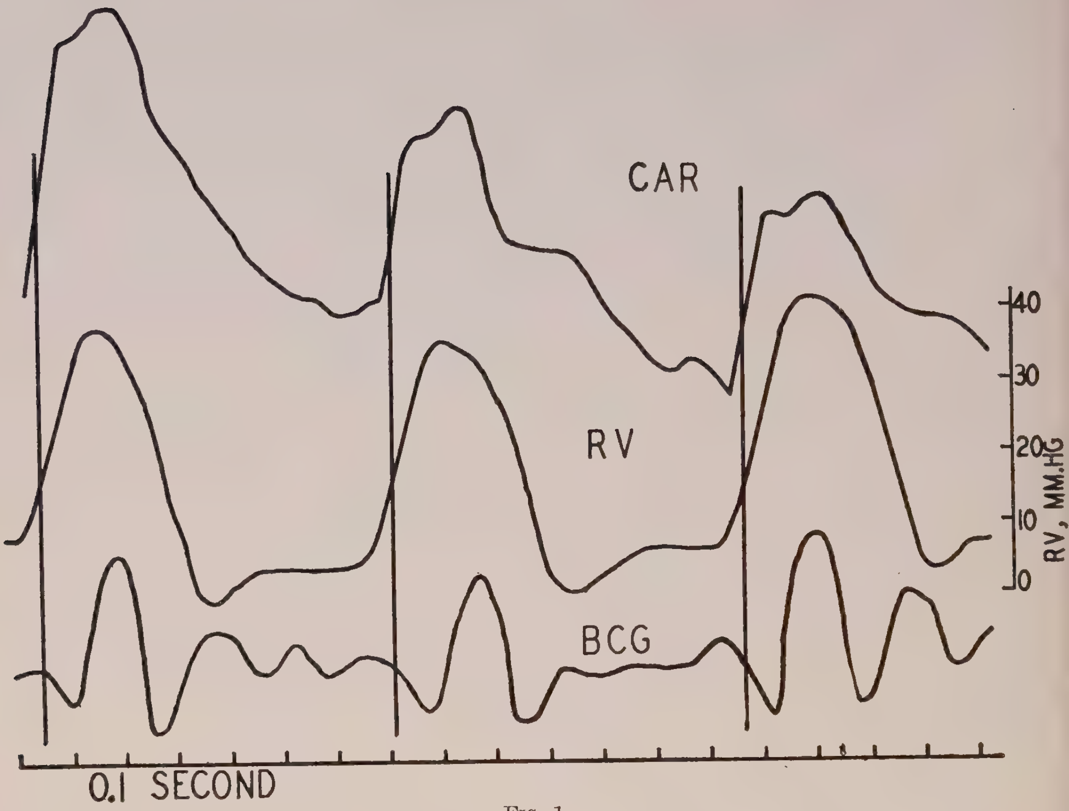

Fig. 1.
Reconstruction of a simultaneous record of carotid surface pulse, right ventricle pressure, and ballistocardiogram.

ings[9] stating that, in the human, a time discrepancy between the start of ejection from the 2 ventricles exists in almost all cases. The measurements were taken from electrokymograph recordings. In this series, either ventricle might precede, whereas in our series, the left ventricle usually precedes the right. The time discrepancies are somewhat larger in our series. Whether these differences are attributable to the small number of cases reported here remains to be determined.

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[9] Chamberlain, W. E., Boone, B. R., Ellinger, G. F., Henny, G. C., and Oppenheimer, M. J., *Fed. Proc.*, 1947, **6**, 88.

15930

Lytic Effect of Bacterial Products on Lymphocytes of Tuberculous Animals.

CUTTING B. FAVOUR. (Introduced by R. J. Dubos.)

From the Laboratories of the Rockefeller Institute for Medical Research, New York.

Introduction. Buffy coats or splenic explants from tuberculous guinea pigs[1] or tuberculous rabbits[2] are killed when grown in concentrations of tuberculin which are harmless for normal animal tissue explants. This phenomenon is not a manifestation of anaphylaxis, for tissue culture explants from animals sensitized to horse serum are not injured by horse serum added to the cultural fluid.[3] The cytotoxic effect is disease specific, however for tuberculin will not injure tissue culture explants from guinea pigs infected with the streptococcus which causes chronic lymphoadenitis in the guinea pig, whereas a soluble protein derived from the homologous streptococcus will cause cell death in tissue culture explants from guinea pigs showing the delayed type of skin hypersensitivity to streptococcal protein.[4] These studies have been done with explants composed of a variety of cell types and observations have been made after 18 hours in order to measure the degree of cellular migration as well as change in cell morphology. The present report is a restudy of the cytotoxic effect of tuberculin on tissue cultures derived from tuberculous animals. The methods used differ from those previously reported in that the explanted cells have been studied in suspensions instead of in clots; quantitative measurements on the different cell types in the suspensions have been carried out and the time of the observations has been shortened to 2 hours or less. These modifications in technic have made it possible to evaluate the effect of tuberculin on the separate cell types present and to suggest the possible role which some of these cells play in the tuberculin reaction.

Experimental. (1) *Mouse Tuberculosis* was produced by inoculating 6-week-old albino mice intravenously with 0.1 cc of a 6- to 10-day culture of H37Rv grown in a synthetic media previously described.[5,6] Eighty per cent of the animals died within 3 to 6 weeks of progressive tuberculosis. Those surviving at 3 to 4 weeks were selected for experiments.

(2) *Mouse Paratyphoid* with a similar mortality to that of mouse tuberculosis was produced in comparable mice with 0.05 cc of 10^{-7} 6-hour broth culture of *Salmonella enteriditis* given subcutaneously. Acutely ill animals surviving at 3 to 4 weeks were chosen for experiments.

(3) *Guinea Pig Tuberculosis* was produced in albino 350 g animals with 0.1 mg of H37Rv grown on an egg slant and introduced into the groin of the animal. Guinea pigs surviving at 2 to 6 months were chosen for experiments.

Three different preparations of tuberculin had the same effects when used in amounts of 50 or 400 γ per cc; PPD-s,[7] PPD 67-2* and a crude tuberculin prepared in our laboratory and used in a dilution of 1:1000. The supernatant of a heat-killed (8 minutes 63°C) 8-hour broth culture of *S. enteriditis* in a final dilution of 1:200 served as the paratyphoid counterpart to tuberculin.

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[1] Rich, A. R., and Lewis, M. R., *Bull. Johns Hopkins Hosp.*, 1932, **50**, 115.

[2] Heilman, D. H., Feldman, W. H., and Mann, F. C., *Am. Rev. Tub.*, 1944, **50**, 344.

[3] Meyer, Kurt, and Loewenthal, H., *Z. f. Immunitatsforsch. u. exp. Therap.*, 1927, **54**, 420.

[4] Moen, J. K., and Swift, H. F., *J. Exp. Med.*, 1936, **64**, 339.

[5] Dubos, R. J., and Davis, B. D., *J. Exp. Med.*, 1946, **83**, 409.

[6] Pierce, C., Dubos, R. J., and Middlebrook, G., *J. Exp. Med.*, July, 1947, in press.

[7] Seibert, F. B., and Glenn, J. T., *Am. Rev. Tub.*, 1941, **44**, 9.

* Kindly supplied by J. McCarter. Molecular weight about one-half that of PPD-s.

Suspensions of guinea pig cells consisting of 60 to 95% lymphocytes, 2 to 6% monocytes and 1 to 30% granulocytes were prepared from single or pooled blood samples (10 to 30 cc). Resuspended buffy coats from such bloods were allowed to stand for 10 to 20 minutes until clumped granulocytes had largely settled out. The supernatants were layered over a solution of concentrated bovine albumin similar to that used by Ferrebee and Geiman[8] for the concentration of malarial parasitized red blood cells. By pilot centrifugations of cell suspensions from tuberculous and control animals it was found that albumin with a specific gravity of approximately 1.072-1.078 would permit the red cells only to pass the plasma-albumin interface. This specific gravity was most conveniently produced by diluting 4 parts of 30% commercial crystalline bovine albumin solution[†] with one part of distilled water or physiological saline. It is important that the albumin salt concentration, the pH and the centrifuge tube size which are recommended by Ferrebee and Geiman be used. Centrifugation was done at 600-1000 r.p.m. in a horizontal head (16 cm radius) centrifuge for 5-15 minutes until by visual inspection the buffy coat had reached the plasma-albumin interface. Thereafter, the tubes were spun at 1200-1500 for 15-30 minutes until the red cells were well down into the albumin.

Suspensions of mouse spleen cells consisting of 45-80% small mature lymphocytes, 30-50% acute splenic tumor cells[9] and 2-8% granulocytes were prepared from minced spleen pulp. The cells were suspended first in 10% normal serum in saline containing 200 mg % glucose and 1 mg heparin per cc. The gross clumps of cells were allowed to settle for 20-30 minutes, the supernatant was washed twice by centrifuging and resuspending in 10% serum saline-glucose solution and

TABLE I.

In Vitro Effect of Tuberculin on Spleen Cells from a Tuberculous Mouse.

	Time	WBC	Band forms		Segmented forms		Lymphocyte		Splenic tumor cells		Monocytes		Eosinophiles		Basophiles	
			%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.
Control	5′	13,230	3.8	50	3.1	41	40.8	5410	50.3	6650	1.5	20	0.3	40	0.2	27
	60′	12,680	3.4	43	2.2	28	25.8	5800	47.2	5975	1.2	15	—	—	0.2	25
50 γ/cc PPD-s	5′	15,890	5.3	84	4.7	75	43.6	6920	44.8	7130	1.5	24	0.1	16	—	—
	60′	12,680	5.6	71	5.6	71	24.8	3042	62.0	7860	1.8	23	—	—	0.2	25

Note: A 56% lympholysis is unaccompanied by loss of other cell types present.

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[8] Ferrebee, J. W., and Geiman, Q. M., *J. Inf. Dis.*, 1946, **78**, 173.

[†] pH 7.3, freezing point —0.64°C, specific gravity 1.087 at 20°C, obtained from Dr. J. B. Lesh, Armour Laboratory, Stockyards, Chicago, Illinois.

[9] Rich, A. R., and Lewis, M. R., *Bull. Johns Hopkins Hosp.*, 1932, **50**, 134.

finally it was suspended in whole normal serum for *in vitro* studies. The few red blood cells present did not interfere with subsequent cell studies. Nonspecific cell destruction during manipulations was minimized by maintaining the blood and spleen cells under oil (Bayol F) in the presence of 200 mg % glucose and under an atmosphere of 5% CO_2 in air.

Cytotoxic effects were demonstrated by adding 0.2 cc of cell suspensions (12,000 to 30,000 per cu mm of fresh serum) to 0.2 cc of bacterial extract in 0.5% albumin in saline. There was no difference between homologous or normal serum or plasma as a suspending fluid. The lytic effect of bacterial substances was measured by careful white counts (5-10% error) and differential counts (1-2% error on 1000 cells counted). Sample protocols illustrate the results of the method (Table I and II).

Results. (1) A portion of the small mature lymphocytes from blood or spleen of tuberculous mice are specifically lysed by tuberculin.

(2) Lymphocytes from normal mice or from mice infected with *S. enteriditis* (SE) are not lysed by tuberculin.

(3) Lymphocytes from mice infected with SE are specifically lysed by soluble substances (SESS) derived from cultures of SE.

(4) Lymphocytes from normal or tuberculous mice are not lysed by SESS.

(5) In mice 20-50% of the lymphocytes are specifically lysed. Lysis is complete within 20 to 60 minutes and does not progress thereafter at a greater rate than that induced by the trauma of the procedure (1-10% in 2 hours).

(6) Lympholysis can be blocked by adequate amounts of phosphate buffer at pH 7.2. It can also be blocked or greatly inhibited by suspending the cell system in 0.5% bovine albumin in saline, or in saline alone instead of fresh serum.

(7) Mouse granulocytes, monocytes and acute splenic tumor cells are not affected by the specific lytic agent before or after lympholysis.

(8) A similar specific lympholysis occurs

TABLE II.

In Vitro Effect of Tuberculin on White Cells from a Tuberculous Guinea Pig.

			Differential count											
	Time	White cell count	Band forms %	Band forms No.	Segmented %	Segmented No.	Lymphocyte %	Lymphocyte No.	Monocyte %	Monocyte No.	Basophile %	Basophile No.	Eosinophile %	Eosinophile No.
Control	5′	3900	1.4	56	29.8	1162	61.1	2381	5.7	222	—	—	1.9	74
	60′	3672	0.6	22	28.4	1042	64.8	2350	5.3	195	—	—	1.6	59
	90′	3892	0.8	31	31.2	1222	61.4	2383	4.9	190	—	—	1.7	67
50 γ/cc PPD 67-2	55′	4300	0.6	26	29.4	1264	61.9	2662	6.5	280	0.2	8	1.4	60
	60′	3520	0.3	11	36.0	1268	56.9	2001	4.6	162	0.3	11	1.9	67
	90′	2320	0.4	9	18.8	436	73.8	1713	5.0	116	—	—	2.0	46

Note: At one hour no granulocyte lysis; 25% lympholysis.
At 90 minutes, 66% neutrophile lysis and 36% lympholysis.

in blood from tuberculous guinea pigs. It also can be blocked by phosphate buffer or in complement-poor suspending fluids.

(9) Guinea pig lympholysis is followed by destruction of granulocytes and monocytes as well as some of the remaining lymphocytes. If lympholysis is blocked, all cell types remain similarly unaffected.

(10) Specific lympholysis can also be demonstrated in fresh heparinized mouse or guinea pig blood to which PPD is added directly. The degree of lysis and nature of the phenomenon are the same as seen in more homogenous cell systems; the leucopenia and lymphopenia present in tuberculosis, however, complicate accurate cell counts.

15931 P

Inhibition of Histamine Whealing in Human Skin by Pyribenzamine Hydrochloride Using Iontophoretic Technique.

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It is well known that histamine produces a wheal in the skin of man, whether introduced by injection or by iontophoresis. Inhibition of these wheals has been accomplished by means of the newer histamine antagonists when they are administered orally or by injection; the reaction of the skin to the subsequent intradermal injection of histamine is thereby diminished.[1-3] In the present study various substances were introduced into the skin by iontophoresis. Their influence upon the development of wheals subsequently produced by the iontophoretic administration of histamine into the same sites was then observed.

Method. An ordinary iontophoresis apparatus was used. The current density in all cases was 0.5 milliamperes per square centimeter. Canton flannel saturated with the solutions to be tested was applied to the skin. The positive electrode applied to the flannel was made of aluminum foil. The time of the application of the electrode to the skin was 3 minutes. Each of the test solutions was introduced by iontophoresis into a rectangular area of skin of the ventral surface of the forearm. Immediately after the removal of the electrode the area was cleansed with distilled water. Erythema and papules were usually observed at the site following the introduction of the initial substance. When the area regained its normal appearance histamine was introduced into the skin in the manner described using a rectangular electrode which was superimposed on the initial site as a vertical bar in the form of a "T." This was done in order to compare readily the whealing effect of histamine in the normal skin with its effect upon the immediately adjacent prepared skin.

Experimental. Solutions of pyribenzamine hydrochloride were made up in concentrations of 10, 5, 1 and 0.1%. Each of these solutions was introduced into the skin by iontophoresis. Following injections of these concentrations, the subjects usually felt a slight local burning sensation which subsided a few minutes after the electrode was removed. Initially there was an erythema at the site and papules formed about the pores. In all cases it required approximately one and a half hours for this reaction to disappear completely. The dilutions of histamine phosphate used were 1 to 10,000 and 1 to

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[1] Friedlander, S., and Feinberg, S., *J. Allergy*, 1946, **17**, 129.

[2] Arbesman, C. E., Koepf, G. F., and Miller, G. E., *J. Allergy*, 1946, **17**, 203.

[3] Cohen, M. B., Friedman, H. J., Burke, M., and Abram, L. E., *J. Allergy*, 1947, **18**, 32.

100,000 in distilled water. The whealing normally produced by either of these dilutions in 10 different subjects was essentially inhibited in every case when applied to sites previously prepared with 10 and 5% pyribenzamine hydrochloride, the inhibition being most complete with the dilute histamine. Low papules were noted about the sweat glands. Around the pyribenzamine site the flare was inhibited but there was extreme erythema at the site. Lesser inhibition of the wheal was noted consistently with lower concentrations of pyribenzamine, *i.e.,* 0.1 and 1%. Also the degree of inhibition appeared to depend upon the reactivity of the individual skin to both the histamine and to the density of electrical current. Similar experiments were performed introducing the histamine 24 hours after the iontophoretic introduction of pyribenzamine solutions into the skin. It was repeatedly found that almost the same degree of inhibition existed after the longer interval of time.

The effects of sodium chloride, ephedrine hydrochloride, ephedrine sulfate and procaine hydrochloride in concentrations of 10, 5, 1 and 0.1% were also studied in the same manner. When any of these substances were introduced into the skin by iontophoresis the subjects noted a slight burning sensation, and on removal of the electrode, there was an initial erythema and papule formation about the pores. This reaction disappeared in all cases in approximately one and a half hours and no further effects were noted. No inhibition of the histamine wheal or flare was observed by any of these substances when using dilutions of histamine phosphate of 1 to 10,000 and 1 to 100,000.

Summary. Using the iontophoretic technic, it was observed that pyribenzamine hydrochloride inhibited histamine wheal formation in the human skin whereas sodium chloride, ephedrine hydrochloride, ephedrine sulphate and procaine hydrochloride failed to produce this effect in the concentrations used.

The Pyribenzamine Hydrochloride was kindly supplied by the Ciba Pharmaceutical Products Corporation.

15932

A New Bone Deformity in the Chick.*

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A bone deformity has been observed in White Leghorn cockerels which were raised on purified diets. The first observation was made on chicks receiving diet 653 of Hill, Norris, and Heuser,[1] to which had been added 55 μg of synthetic folic acid† per 100 g of diet. The incidence of the malformation was relatively low, occurring in approximately 15 to 20% of the chicks. The weights of the affected chicks at 6 weeks of age were 150 to 250 g below the average weight (460 g) of the unaffected chicks. The most severe cases had difficulty in walking, and their legs splayed out from the tibio-femural joint. No slipping of the tendon from the condyle

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* This work was undertaken in cooperation with the Office of Naval Research, Navy Department, Washington, D.C., and was aided by grants to Cornell University by the Commercial Solvents Corp., New York, N.Y., the Cooperative G.L.F. Exchange, Inc., Ithaca, N.Y., and the Nutrition Foundation, Inc., New York, N.Y. The work was conducted in the Nutrition Laboratories of the Department of Poultry Husbandry. We are indebted to Miss Florence A. Farmer for technical assistance.

[1] Hill, F. W., Norris, L. C., and Heuser, G. F., *J. Nutrition,* 1944, **28**, 175.

† We are indebted to Lederle Laboratories, Pearl River, N.Y., for synthetic folic acid.

TABLE I.
Average Body Weight of 6-weeks-old Chicks Together with Measurement of Bone Deformity.

Diet	Wt 6 wks g	Visual incidence of deformity %	Avg angle of tibiae °	Significance of difference
Normal diet: corn, wheat, soybean, fish meal	537 (15)*	0	45.8 (12)	—
Basal diet	197 (14)	50	57.7 (14)	1666:1
” ” + 10% fish meal	267 (15)	40	54.0 (15)	9999:1
” ” + 4% gelatin†	326 (15)	33	51.9 (13)	555:1
” ” + 4% gelatin + 10% fish meal	465 (15)	6.7	48.5 (15)	27:1

* Numbers in parentheses indicate the number of chicks represented in the averages.
† When gelatin was added the level of casein was reduced from 26% to 23%.

of the tibio-metatarsal joint as occurs in perosis was observed. The feathers of the abnormal chicks were very rough due to the use of the wings for propulsion. The hemoglobin levels of both groups were normal.

The length, weight, density and breaking strength of the metatarsi, and bone ash and degree of twisting of the tibiae were ascertained for both deformed and normal chicks. The degree of twisting was determined by placing the 2 articular elevations of the distal end of the tibia on a flat surface and measuring the angle made with the horizontal surface by a line drawn from the point of contact through the widest part of the expanded proximal end. The normal and deformed bones are shown in Fig. 1, which also illustrates the angle just described. A statistical analysis of the data showed that the density of the deformed metatarsi was significantly lower than that of the normal bones, and that the difference in degree of twisting of the normal and deformed tibiae was highly significant. There was no difference in bone ash, nor were there any differences in the other measurements that could not be attributed to differences in body size and weight.

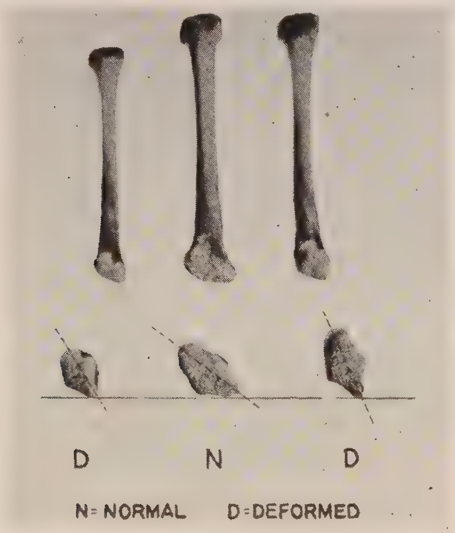

FIG. 1.
Twisting of tibiae in deformed chicks.

In further work gelatin was omitted from diet 653 and 26% alcohol extracted casein was substituted for 27.5% acid-washed casein. Glycine, *l*-cystine and folic acid were added at the levels of 0.7 g, 0.2 g and 100 μg per 100 g of diet, respectively. Crude gelatin and fish meal were added as supplements to this basal diet. Chicks which received a diet consisting of 30% wheat, 30% corn, 30% soybean meal, 5% fish meal, minerals and vitamins showed none of the visual symptoms associated with this deformity and were considered normal. The angle of twisting was determined on the right tibia of all chicks.

The results of this study are presented in Table I, together with a statistical analysis of the data. The incidence of the bone deformity and the degree of twisting at 6 weeks of age were much greater among chicks fed this basal diet than among those fed diet 653. The addition of 4% gelatin or 10% fish meal to this basal diet caused some decrease in the incidence and severity of the deformity. However, the addition of fish meal and gelatin simultaneously was more effective in the prevention of this malformation than the addition of each alone.

The basal diet used in this study was deficient in one or more factors as indicated by the low growth response as well as the occurrence of the bone deformity described. Some of the beneficial effect of the added fish meal and gelatin may have been due to the glycine and/or arginine content, but not all, since the addition of 7.5% gelatin in the previous study did not completely prevent the occurrence of this bone deformity, nor did it allow maximum growth response. This diet contained adequate amounts of the required minerals and known vitamins. The diet also contained 100,000 units of strepogenin per 100 g, which is in excess of that found necessary for growth by Scott, Norris, and Heuser.[2]

Summary. A new bone deformity which is characterized by a twisting of the tibia at the proximal end has been observed in chicks fed purified diets. Under the conditions of this experiment gelatin and fish meal decreased the incidence and severity of the malformation. The diet used appears to be deficient in an unknown factor or factors necessary for maximum growth and the prevention of the bone deformity.

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[2] Scott, M. L., Norris, L. C., and Heuser, G. F., *J. Biol. Chem.*, 1947, **167**, 261.

15933

The Effect of Ingested Mineral Oil on Plasma Carotene and Vitamin A.

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Due to the shortage of biological fats and oils during the war, the use of mineral oil in foods, in the form of imitation mayonnaise or salad dressing replacing the usual food oils, increased considerably. Such food substitutes are also used extensively in weight-reduction diets.

There are numerous reports concerning the potentially harmful effects of mineral oil based upon its interference with the absorption of fat-soluble vitamins,[1-8] particularly vitamin A and its precursor, carotene.[1-6] Most of the work is based on animal experiments in which huge amounts of mineral oil were used compared with what would ordinarily be taken by man. Nevertheless, the Council on Foods and Nutrition of the American Medical Association concluded[9] that "the indiscriminate use of mineral oil in foods and cooking is not in the interest of good nutrition, and any such use should be under careful supervision of a physician."

Curtis and his collaborators[10,11] showed in man that the ingestion of mineral oil will prevent blood carotene rises consequent to simultaneous intake of carotene-rich foods. They[11] also reported a decrease in blood carotene in subjects on a normal diet who took 20 cc of mineral oil immediately before

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[1] Burrows, M. T., and Farr, W. K., PROC. SOC. EXP. BIOL. AND MED., 1927, **24**, 719.

[2] Dutcher, R. A., Ely, J. O., and Honeywell, H. E., PROC. SOC. EXP. BIOL. AND MED., 1927, **24**, 953.

[3] Dutcher, R. A., Harris, P. L., Hartzler, E. R., and Guerrant, N. B., *J. Nutr.*, 1934, **8**, 269.

[4] Mitchell, H. S., PROC. SOC. EXP. BIOL. AND MED., 1933, **31**, 231.

[5] Jackson, R. W., *J. Nutr.*, 1934, **7**, 607.

[6] Curtis, A. C., and Horton, P. B., *Am. J. Med. Sc.*, 1940, **200**, 102.

[7] Smith, M. C., and Spector, H., *J. Nutr.*, 1940, **20**, 19.

[8] Elliott, M. C., Isaacs, B., and Ivy, A. C., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 240.

[9] Council on Foods and Nutrition, *J. A. M. A.*, 1943, **123**, 967.

[10] Curtis, A. C., and Kline, E. M., *Arch. Int. Med.*, 1939, **63**, 54.

[11] Curtis, A. C., and Ballmer, R. S., *J. A. M. A.*, 1939, **113**, 1785.

2 or 3 meals daily; if the mineral oil was saturated with carotene at body temperature, it did not produce any change.

Since these authors observed the effects only over a period of 15 days and since spontaneous daily fluctuations in blood carotene content are frequently observed, it was deemed desirable to make observations over a longer interval. Blood vitamin A levels also were determined simultaneously.

Twenty-five subjects, comprising 5 groups, were studied.

Group I—3 normals taking no form of mineral oil.

Group II—6 normals taking 1 tablespoonful of Leanermaise* with noon and evening meals daily.

Group III—4 normals taking 1½ teaspoonfuls of Leanermaise* with noon and evening meal daily.

Group IV—3 normals taking 1 tablespoonful of mineral oil with noon and evening meal daily.

Group V—9 patients with psoriasis vulgaris† of whom 6 ingested 1 tablespoonful of mineral oil with noon and evening meals and 3, 1 tablespoonful with each of 3 meals.

The diet of all subjects was unrestricted. The mineral oil or salad dressing was taken immediately before or during the meal. Plasma carotene and vitamin A concentrations were determined by the method of Kimble[12] before, and at frequent intervals during, the observation periods of from 3 to 8 weeks.

Results and Comment. The results (Table I) indicate that, concomitant with the prolonged daily ingestion of mineral oil either as such or in the form of mayonnaise dressing at meal time, the plasma carotene concentration decreased about 50%. The decline was about the same for those using 2 tablespoonfuls as for those using 3 teaspoonfuls; no difference was evident between those subjects on Leanermaise and those on regular mineral oil. Some decrease was noted within 1 to 2 weeks, and it progressed further in many cases as intake of the oil continued. No significant changes in plasma vitamin A content occurred, and no untoward clinical manifestations appeared.

These alterations are probably due to extraction by mineral oil of food carotene which is thus prevented from entering the body. That extraction was not complete, even in those cases who received 1 tablespoonful of mineral oil with each meal, is indicated by the considerable carotene concentration even after many weeks on this regime.

Since in normal subjects blood carotene parallels the amount of this substance which enters the body[9,13,14] it may be concluded that under the conditions described above mineral oil interferes with the absorption of the provitamin from the gastro-intestinal tract. It is noteworthy, however, that even after prolonged use plasma carotene levels remained substantial, indicating that considerable amounts of the material were still entering the body.

One tablespoonful of mineral oil can theoretically dissolve 70,000 I.U. of carotene (in terms of vitamin A) at body temperature,[11] an amount of the provitamin far greater than that usually provided in one meal (1100-2200 I.U.[9]), or even 3 meals. That the plasma carotene did not fall precipitously to negligible levels in those subjects taking 1 tablespoonful of mineral oil with each meal for 6 weeks indicates either that the extraction of food carotene by mineral oil in the gastro-intestinal tract is far from complete, or that sizable amounts of carotene were ingested between meals, an unlikely possibility which, however, cannot be excluded.

Nevertheless, since from ½ to ⅓ of the vitamin A supply is derived from carotene

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* Generously supplied by Leanermaise Company, Boston, Mass. Composition: 83% mineral oil, 2% fat. According to our analyses, the material used during the first 7 weeks of the study (Lot 1) contained 27 μg vitamin A per g; during the 8th week (Lot 2) it contained 43 μg per g. Both lots were carotene free.

† Subjects under study of vitamin A and carotene metabolism in *Psoriasis vulgaris.*

[12] Kimble, M. S., *J. Lab. Clin. Med.*, 1939, **24**, 1055.

[13] Wald, G., *Am. J. Physiol.*, 1942, **137**, 551.

[14] Brenner, S., and Roberts, L. J., *Arch. Int. Med.*, 1943, **71**, 474.

TABLE I.
Effect of Mineral Oil Ingestion on Plasma Carotene and Vitamin A.

		Plasma carotene—μg per 100 cc									Plasma vitamin A—μg per 100 cc	
			Weeks									
	Subject	Control* (Before min. oil)	1	2	3	4	5	6	7	8	Control* (Before min. oil)	† (After min. oil)
Group I—No mineral oil	1	134	130	153	155	114	118				46	44
	2	132	121	169	150	134	134				53	55
	3	137	125	93	120						48	56
Group II—Leanermaise—2 tbs.	4	124	113	94	88	79	106	83	78	58	36	29
		143									42	
		149									38	
	5	245	204	168	131	144	125	128	125	104	38	38
		215									40	
		225									38	
	6	173	101	96	98	85	87	65			53	47
		162									56	
	7	86	61	46	57	71	65	37			53	46
		115									57	
	8	151	100	112	96	79	72				53	54
		200									63	
	9	153	119	119	104	92	95				38	46
		174									39	
Group III—Leanermaise—3 teaspoons	10	131	121	101	84	62					41	39
		136									43	
	11	97	106	88	95	83	80				35	44
		146									45	
	12	207	136	140	128	104					50	47
		145									45	
	13	164	129	102	114	114	102	113	94		34	33
		179									38	
Group IV—Mineral oil—2 tbs.	14	168	139	145	139	116					33	36
		181									39	
	15	156	118	107	101	83					26	31
		144									34	
	16	159	137								56	59
		161									32	
Group V—Mineral oil—3 tbs.	17	232			91			129			64	60
	18	166			91			77			52	48
	19	72				26					33	30
Mineral oil—2 tbs.	20	111		76			71				66	35
	21	109			106				77		60	46
	22	187	143		108	62					47	36
	23	85		76			99			65	37	72
	24	95				50					65	63
	25	134		102		125		108			63	61

* Multiple values given in this column represent determinations spaced one week apart.

† The value given in this column is the last one obtained during the observation period which is the same number of weeks as that indicated for carotene. Intervening values are omitted for the sake of brevity.

in the usual American diet it must be presumed that the use of mineral oil under the conditions described above precludes entrance to the body of sizable amounts of vitamin A in the form of its precursor. In subjects with already restricted diets, such a loss may become clinically significant. If, however, the diet provides sufficient excess of carotene or vitamin A to meet this loss no derangement might occur.

The unchanged vitamin A levels are not surprising since it is well known[13,14] that the concentration of this vitamin in the blood remains relatively constant for a very long time

(6 months) in normal individuals who are subjected to restricted vitamin A and carotene intake. Therefore, no conclusion can be drawn on our subjects with regard to the effect of mineral oil on the supply of vitamin A as such to the body. Experiments on animals indicate that mineral oil interferes far less with absorption of vitamin A than carotene.[3,5,9,15] Furthermore, mineral oil containing vitamin A provides a ready source of this vitamin to vitamin A-deficient animals.[6] Similar experiments on man are contemplated.

Summary. Continued ingestion of mineral oil as such or in the form of mayonnaise dressing by 20 subjects on a normal unrestricted diet resulted in a moderate decrease in plasma carotene concentration. Vitamin A levels remained unchanged.

It is concluded that simultaneous ingestion of mineral oil with food prevents substantial amounts of food carotene from entering the body.

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[15] Rowntree, J. I., *J. Nutr.*, 1931, **3**, 345.

15934

Nutritional Requirements of *Tritrichomonas foetus* with Special Reference to Partially Digested Proteins.

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The purpose of this group of experiments is to review the metabolism of the endozoic parasite, *Tritrichomonas foetus,* in order to determine if this organism requires the same general type of nitrogen as that required by other animals, and also to determine the effects of partially-digested proteins on the growth rate of this organism. It is believed that this is the first more or less detailed study of protein nutrition, using synthetic media, in a bacteria-free endozoic protozoan which does not inhabit the blood.

Several methods have been proposed for the cultivation of *Tritrichomonas foetus*. A medium commonly used is egg-serum-Locke's medium (ESL). Collier and Boeck's modification[1] was used in this experiment for the maintenance of the culture. The test tubes used were 4″ x ⅜″ and were filled with 2 cc of liquid medium. After the media tubes had been prepared, they were each inoculated with approximately 380 organisms, this number being obtained from 2 bacteriological loopfuls of a well-mixed, 5- to 7-day-old, ESL culture. All culture tubes were incubated at 25°C in an oven with a saturated atmosphere to prevent evaporation as far as possible. Maximum growth in an ESL culture was obtained in 5 to 7 days.

The method of counting used throughout this experiment is a very simple one. The test tube is shaken thoroughly. Then a bacteriological loopful of liquid is removed aseptically to a clean slide. The number of organisms present in the center of the drop for one ocular field is counted. This procedure is followed 3 consecutive times to insure reasonable accuracy in a minimum amount of time. This method has been calculated statistically to be accurate with a maximum error of ±5%. Two cc of liquid were maintained as the standard volume in all media so that the figures obtained by this counting method in different media would be comparable. Bacteria-free cultures were maintained throughout the experiment.

Three standard media were compared to determine the differences in growth response in dissimilar types of media. Broth-serum-glucose (BSG) described by Andrews and

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[1] Collier, J., and Boeck, W. C., *J. Parasit.*, 1926, **12**, 131.

[2] Andrews, J., and von Brand, T., *Am. J. Hyg.*, 1938, **28**, 138.

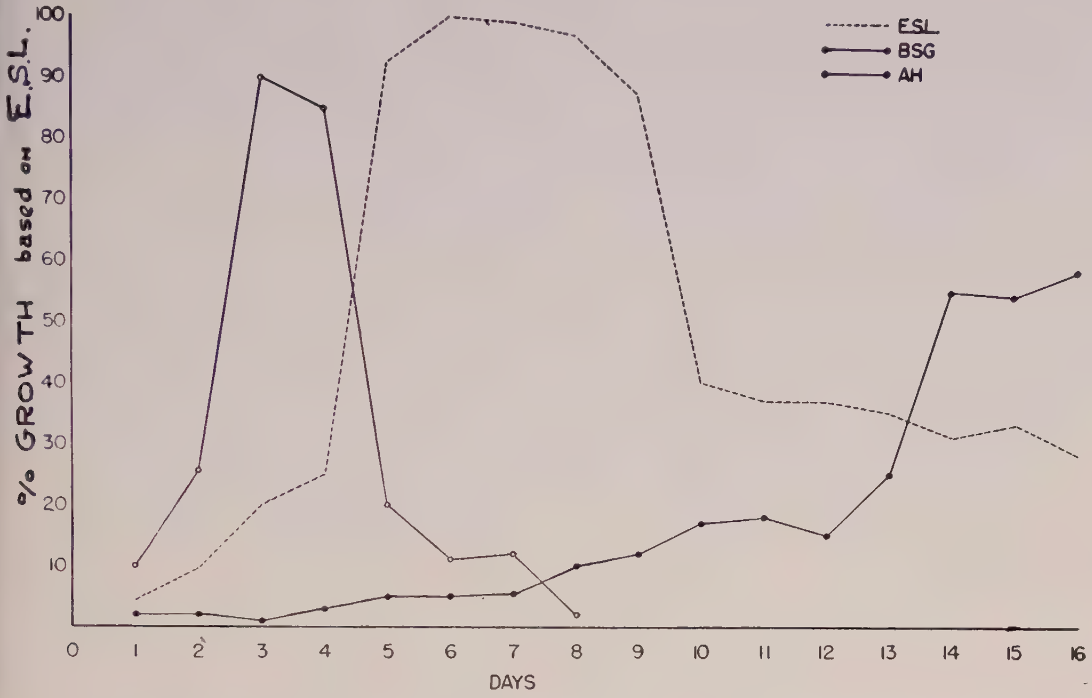

FIG. 1.
Comparison of growth results in Egg-serum-Locke's (ESL), Agar serum (AH), and Broth-serum-glucose media (BSG).

von Brand,[2] agar-serum (AH) used by Weinman[3] and ESL, previously described, were used to represent the 3 types. Fig. 1 shows the comparison between these 3.

Fig. 1 shows that growth in AH is considerably slower than in either of the other media; this probably is due to the high viscosity of the agar. High viscosity is known to inhibit division of organisms.[4] Growth in BSG, it is observed, reaches its peak rapidly and falls in the same manner. A tentative explanation for this in the light of subsequent experiments on digested proteins is that this medium, which is composed of peptones and partially-digested beef proteins, is able to initiate growth better than whole proteins and yet is in some way incomplete.

Emphasis in recent research has been placed on the amino acid requirements of many organisms. The requirements of *Tetrahymena geleii,* a free-living ciliate, have been meticulously worked out by Kidder and Dewey.[5] The same general technics have been used in this analysis of the requirements of *Tritrichomonas foetus.*

The complete amino acid medium of Kidder and Dewey[5] was used in these experiments.

Changes have been made in tryptophane (1— to dl), lysine (dl to 1+), glutamic acid (dl to 1+). In these cases the mg % was varied to insure an equal amount of the active form. The complete medium was supplied from the laboratory of Dr. M. S. Dunn, Department of Chemistry, U.C.L.A. The amino acids were prepared in Dr. Dunn's laboratory. The entire solution was sterilized in amounts of 2 cc per test tube, and the test tubes were then inoculated with a standard ESL culture of 5 days' incubation. Three subsequent transfers were made 5 days apart to be sure that none of the ESL medium carried over in the original transfer was affecting the results.

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[3] Weinman, D., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 82.

[4] Hegner, R., and Eskridge, L., *J. Parasit.,* 1936, **22**, 223.

[5] Kidder, G. W., and Dewey, V. C., *Physiol. Zool.,* 1945, **18**, 136.

TABLE I.
Growth Results When One Amino Acid Is Omitted from the Complete Amino Acid Medium. Medium does not contain serum.

Amino acid omitted	% growth based on ESL
Alanine	87.84
Arginine	0
Aspartic Acid	84.26
Cystine	83.9
Glutamic Acid	85.7
Glycine	0.6
Histidine	1.08
Hydroxyproline	84.8
Isoleucine	6.0
Leucine	2.4
Lysine	0.6
Methionine	0
Phenylalanine	0
Proline	2.4
Serine	0
Threonine	0.6
Tryptophane	0
Tyrosine	83.8
Valine	1.62
None omitted	85.9

TABLE II.
Comparison of Growth Obtained in the Complete and Basic Amino Acid Media with and without the Inclusion of Serum.

	% growth based on ESL			
	Days			
Medium	4	6	8	10
ESL	25	100	97	40
Comp. A.A.				
—with serum	52	101	101	90
—no serum	50	85.5	81	77.5
Basic A.A.				
—with serum	40	86	85	60
—no serum	40	76	75	61

To ascertain the amino acids essential for the growth of *Tritrichomonas foetus*, each amino acid was removed individually from the complete medium. Table I gives the percentage growth based on ESL for each amino acid omitted. Maximum average growth in ESL at 6 days was taken arbitrarily as 100%. These data are based on the 3rd transfer and give the percentage maximum growth obtained regardless of the day on which this was obtained. In the case of those amino acids judged dispensable, the time of maximum growth ranges from the 5th to the 7th day.

The amino acids which can be thus declared essential for the growth of this organism are arginine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophane, and valine. It will be noted that a negligible amount of growth occurs in the absence of certain essential amino acids; however the number of organisms obtained in these tubes is insignificant according to the counting method used.

There are various possible explanations for this slight growth in the absence of some essential amino acids. Because of the high purity of the amino acids tested, it is doubtful if contamination as reported by other workers[5,6] plays a role here. A more probable explanation is that *Tritrichomonas foetus* is able to carry on a slow synthesis of certain amino acids. This has been shown to occur in comparable experiments with Lactobacilli.[7]

After determining the amino acids essential to the continued growth of this organism, an attempt was made to establish a vigorous culture on these amino acids alone. Growth in such a medium was 10% lower than that found in the complete amino acid medium. Results such as these have been observed by other workers on different organisms although the cause has not yet been ascertained.[5,8,9] Table II compares growth in the complete and in the basic amino acid media. The improvement in the media on the addition of blood serum indicates that serum may contain an unknown factor acting as a growth stimulant.

Once the required amino acids for *Tritrichomonas foetus* were determined, the growth response of the organism in proteins and in partially-digested protein media was studied. All the protein media were prepared with a sufficient quantity of glucose, vitamins, and other growth stimulants so that any limiting effect would be due for the most part to the protein itself. Previous ex-

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[6] Hegsted, D. M., and Wardwell, E. D., *J. Biol. Chem.*, 1944, **153**, 167.

[7] Dunn, M. S., Shankman, S., Camien, M. N., and Block, Harriette, *J. Biol. Chem.*, 1947, **168**, 1.

[8] Hegsted, D. M., *J. Biol. Chem.*, 1944, **156**, 247.

[9] Snell, E. E., and Guirard, B. M., *Proc. Nat. Acad. Sci.*, 1943, **29**, 66.

periments have shown that 0.2% glucose was more than sufficient to satisfy the glucose requirements of *T. foetus*.[2] Although the addition of vitamins to ESL, BSG, or AH in this laboratory had no appreciable effect on growth, vitamins were added to all protein and hydrolysate media in the concentration suggested by Kidder and Dewey.[5] In this experiment the proteins used were casein, lactalbumin, and wheat germ protein. Attempts to use soy bean protein were unsuccessful and gave variable results.

Casein. 1. Vitamin-free casein (Biological Materials Co., Ohio) was mixed with sufficient water to form a paste (approximately 30% protein by analysis). Test tubes were filled with this paste as in the preparation of crumbled egg slants and were overlaid with the standard solution consisting of Locke's medium with added glucose, vitamins, and sterile serum. No growth was obtained, undoubtedly because of the low pH involved. The per cent protein was determined in all cases by a nitrogen analysis on the undiluted protein, determined by the semimicro titration total-nitrogen method.

2. Technical casein was solubilized with NaOH to bring it to a pH of 7. The insoluble casein was filtered off and the supernatant diluted to obtain a 1.5% protein solution by analysis after the addition of the necessary salts, vitamins, and carbohydrates. Some slight growth was obtained.

3. It is believed that drum drying as used in the preparation of technical casein may be detrimental to the protein molecule in a way which may change the biological value of the protein irreversibly. Casein precipitated from skim milk was prepared in the laboratory by the method of Van Slyke and Baker.[10] This casein is relatively ash-free, contains little calcium phosphate, and is readily soluble in dilute alkali. Growth in a 1.5% protein solution of this kind was 10% higher than growth in technical casein solutions at the same pH.

4. Rennin precipitation of milk proteins yields paracasein, a protein sometimes assumed to be the first product in the proteolytic digestion of casein. The paracasein prepared for this experiment by the use of Rennet tablets was not made calcium-free. Growth in this medium was 14% above that obtained in the freshly prepared casein medium.

The preparation of trypsin, pepsin, and papain digests of casein, lactalbumin, and wheat germ protein is shown in Table III. In general the method for preparing the digests is as follows:

The protein solution is made to the desired pH at a concentration of 6% protein. This solution is heated to the required temperature and the necessary amount of enzyme added. Since it was considered undesirable to add a preservative such as toluene during the digestion, the equipment used and most of the materials were first sterilized to keep down as much as possible bacterial contaminants. After the digestion had been carried to the desired stage, the enzyme was inactivated by autoclaving at 15 lb pressure for 5 minutes. Following inactivation, the pH of the digest was raised to pH 7.7 with rapid stirring to dissolve as much of the protein precipitated during the digestion as possible. The digest was then filtered through loose cotton to remove the undissolved protein. As far as can be ascertained the only amino acid precipitate forming after this procedure is tyrosine, a dispensable amino acid for *Tritrichomonas foetus*.

The digest was then made into a medium containing 1.5% of the protein digest, vitamins, glucose, salts, and human blood serum. Two cc of this complete medium were used for each test tube (4″ x ⅜″) and sterilized in the usual manner. Much better growth was obtained in media with the addition of serum. In some cases no growth was observed without serum; in others, although growth was obtained, the amount was insignificant. This stimulatory action of added serum had been noted also in the amino acid media.

Lactalbumin was precipitated from whey at pH 6.0 and then redissolved after wash-

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[10] Van Slyke, L. L., and Baker, J. C., *J. Biol. Chem.*, 1918, **35**, 127.

TABLE III.
Preparation and Growth Results in Proteins and Protein Digests.

Substrate	Enzyme	Subs. conc. %	Enzyme conc.	pH	Temp., °C	Hr	% total nitrogen in sol'n	Day of max. growth	% growth based on ESL
Casein—acid precipitated whole protein—								5	27
Casein	Trypsin	6	2%	8	45	6	48.8	7	105
,,	,,	6	1% added every 12 hr until 5%	8	45	72	90.0	6	75
,,	Pepsin	6	Same as above	6	45	72	87.6	7	85
,,	Papain	6	1%	6	45	25	8.7	7	35
Lactalbumin—freshly precipitated whole protein—								6	33
,,	Trypsin	2	2%	8	50	8	53.0	8	102
Lactalbumin	,,	2	1% added every 12 hr until 5%	8	50	48	91.7	5	82
Wheat Germ Protein—freshly precipitated whole protein—								6	20
WG Protein	Trypsin	6	2%	8	50	3	29.7	6	86
,, ,,	,,	6	2%	8	50	6	51.8	5	102
,, ,,	,,	6	2%	8	50	12	67.7	7	98
,, ,,	,,	6	1% added every 12 hr until 5%	8	50	48	83.6	5	69

ing at pH 7.0. Sufficient lactalbumin was not available to make a complete series of digests.

Wheat germ protein was prepared from the whole germ by suspending it in dilute alkali. The soluble protein was then freed from the salts and carbohydrates by acid precipitation. The washed protein was solubilized at pH 7. Growth results in protein and protein digests of casein, lactalbumin, and wheat germ protein are shown in Table III.

Proteins are thought to be differentiated on the basis of the amino acid grouping present within themselves. These groupings are said to make each protein different in structure and in biological value. The proteins used in this experiment are of varying B.V.; the order of high to low is lactalbumin, casein, and wheat germ protein. (Data on biological values were obtained at the Protein Conference at Rutgers University, N. J., 1945). Although the method of measuring B.V. in the protozoan may not be the same as in the metazoan, the value of the protein as a source of certain amino acids can be ascertained. From the results with *Tritrichomonas foetus*, a similar B.V. order is indicated (Table III).

The results obtained in enzyme digests of these proteins can be stated thus: *Tritrichomonas foetus* requires not only the presence of certain amino acids for good growth but in addition responds better to proteins digested to some extent. The response to whole proteins is very low, unmeasurable without the addition of serum. As the protein is digested more and more and in various ways, as the amount of free amino acid as compared with the amount of soluble whole protein becomes greater and greater, as the time of digestion becomes longer, the growth response becomes better until a certain degree of digestion is attained. It will be observed that basically the results are similar regardless of the protein or enzyme used.

The decrease in the maximum growth rate as the digestion of the proteins approaches the amino acid stage indicates perhaps that *Tritrichomonas foetus* needs strepogenin as well as amino acids for normal growth. Woolley[11-13] has demonstrated the role of strepogenin for normal growth in bacteria, rats, mice and chicks.

Summary. 1. The amino acids essential for the growth of *Tritrichomonas foetus* were determined in a medium of known concentration. This flagellate utilizes nitrogen in approximately the same way as do higher organisms. The amino acids essential for *T. foetus* were determined to be arginine, glycine, tryptophane, histidine, isoleucine, leucine, lysine, threonine, methionine, phenylalanine, proline, serine, and valine.

2. Serum contains some factor capable of improving the media tested.

3. Casein, lactalbumin and wheat germ protein were tested for their growth properties. Results indicate the possible use of *T. foetus* to determine the biological values of proteins.

4. With whole protein, growth is relatively low. In digests, growth improves until the percentage of soluble nitrogen reaches 50%. As digestion continues and more nitrogen goes into solution, the growth rate decreases. This decrease may be due to destruction of strepogenin.

Acknowledgment is made to Dr. D. M. Bartlett, Agricultural Station, U.S.D.A., Beltsville, Md., for the culture used; to Dr. P. G. Hoel, Mathematics Department, U.C.L.A., for aid in determining the statistical significance of the method of counting; to Dr. D. Appleman, Agriculture Department, U.C.L.A., for the use of laboratory facilities and also for helpful suggestions and to Dr. M. S. Dunn and co-workers, Chemistry Department, U.C.L.A., who supplied the synthetic media and gave useful advice.

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[11] Sprince, H., and Woolley, D. W., *J. Am. Chem. Soc.*, 1945, **67**, 1734.

[12] Woolley, D. W., *J. Biol. Chem.*, 1945, **159**, 753.

[13] Woolley, D. W., *J. Biol. Chem.*, 1946, **162**, 383.

15935

Effects of Various Sex Hormones on Excretion of Pregnandiol Early in Pregnancy.*

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Smith and Smith[1] reported that the administration of diethylstilbestrol to a patient during pregnancy would increase the excretion of pregnandiol in the urine. They concluded that synthetic estrogens will stimulate steroid metabolism in the placenta increasing the amount of available progesterone. These authors suggested the use of increasing amounts of diethylstilbestrol to prevent the accidents of late pregnancy, beginning with 30 mg per day at the 16th week of the gestation and increasing the daily dose 5 mg each week until the 35th week. Furthermore, it has been recommended by others that large amounts of steroid hormones be administered early in pregnancy to prevent abortion and to treat threatened abortion.

The purpose of the present study is 2-fold: (1) to determine if the sex hormones will increase the production of progesterone by the placenta early in pregnancy and, (2) to study human fetuses from mothers who have received large amounts of these substances in order to establish that no untoward changes have resulted. The second phase of this study is extremely important in view of the changes induced in the reproductive organs of litters of experimental animals by the ad-

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* This work has been done under a grant from the Douglas Smith Foundation for Medical Research of the University of Chicago.

[1] Smith, O. W., Smith, G. V. S., and Hurwitz, D., *Am. J. Obst. and Gynec.*, 1946, **51**, 411.

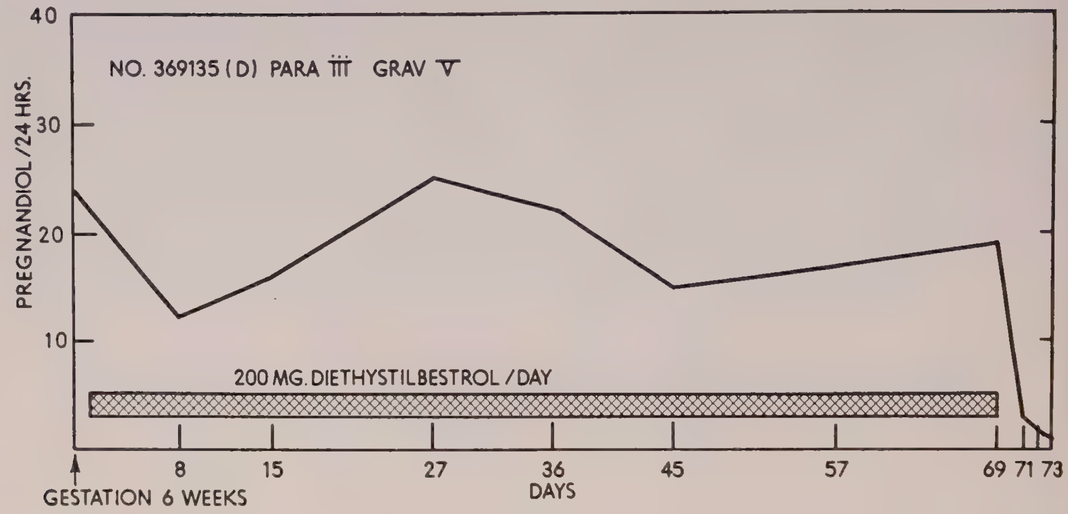

Fig. 1.

Pregnandiol excretion in a patient who had a normal pregnancy. The daily oral administration of 200 mg stilbestrol beginning at 6 weeks and continuing for 69 days did not influence the normal curve. The pregnandiol disappeared promptly at the termination of the pregnancy.

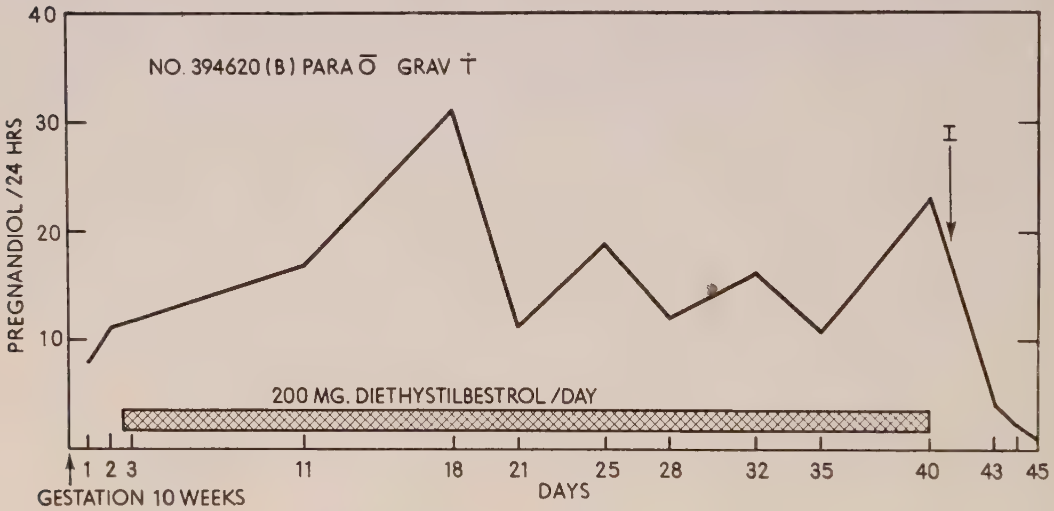

Fig. 2.

The administration of diethylstilbestrol was instituted at 10 weeks gestation and continued for 40 days. No obvious effect on pregnandiol excretion can be seen.

ministration of androgens and estrogens during pregnancy (Greene, Burrill and Ivy[2]) and will be reported separately at a later date.

Methods. This group of patients comprise 15 women with normal gestations. In 7 the pregnancy was interrupted at 12 to 16 weeks because of some serious maternal complication for which therapeutic abortion was advised. One of the patients had a spontaneous abortion at about 14 weeks gestation. The remaining 7 have delivered at term or are continuing their pregnancies.

Pregnandiol determinations were made by a method described in a previous communication.[3] Three hormonal preparations were used, diethylstilbestrol, testosterone proprionate and progesterone. The diethylstilbestrol was administered orally while the other 2 were given in oily solutions by intra-

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[2] Greene, R. R., Burrill, M. M., and Ivy, A. C., *Physiol. Zool.*, 1942, **15**, 1.

[3] Davis, M. E., and Fugo, N. W., in publication.

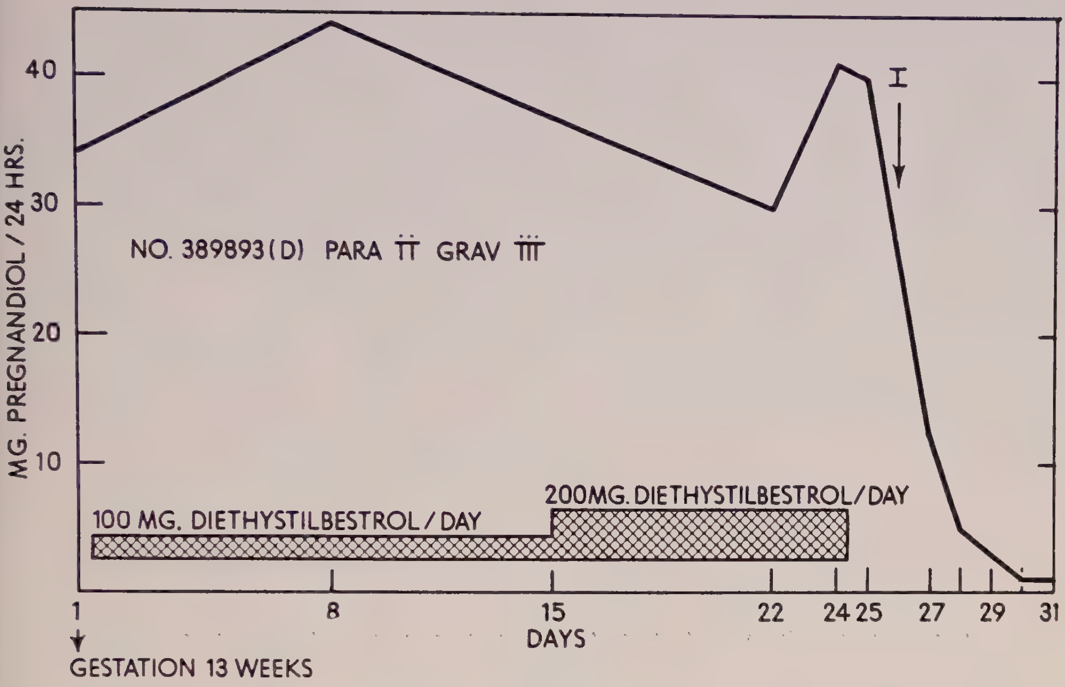

Fig. 3.
Diethylstilbestrol was first administered at 13 weeks of a normal gestation in 100 mg amounts daily for 15 days without effect. The increase in the daily amount to 200 mg did not show any appreciable change.

muscular injections. Several pregnandiol determinations were made in each patient prior to the onset of therapy in order to establish the level of excretion for each individual. The excretion of pregnandiol was followed continuously and at daily or tri-weekly intervals throughout the period during which the hormones were administered and in those patients subjected to therapeutic abortion, until pregnandiol disappeared from the urine. In the remaining cases the determinations were continued throughout pregnancy.

Results. Diethylstilbestrol. Diethylstilbestrol was administered in varying amounts to 10 patients. The administration of this estrogenic substance began at 4 to 10 weeks gestation in most patients and continued for at least 6 or 8 weeks. Most of the patients received 50 mg daily although 2 patients received 100 mg and 2 200 mg. The following figures are representative of the group.

In Fig. 1, 200 mg of diethylstilbestrol was administered daily beginning at 6 weeks gestation (that is about the time of the second missed menstrual period) and continued for 69 days when the pregnancy was terminated by hysterotomy. The size of the fetus was compatible with a gestation of 16 weeks. No change from the normal is apparent in the pregnandiol excretion. The normal rise in the daily output begins at about 15 or 16 weeks gestation. In Fig. 2 a similar amount was administered over a period of 38 days but the onset of therapy commenced at 10 weeks. Pregnancy was again terminated at about 16 weeks and the size of this fetus was comparable to the previous one. There was no appreciable increase in the daily excretion of pregnandiol and the amounts were within normal limits. In Fig. 3, only 100 mg of diethylstilbestrol was administered for a period of 2 weeks, following which the amount was doubled. However, there was no appreciable rise in the amount of pregnandiol excreted although the daily excretion in this individual was considerably greater than in the other 2.

In the entire group of patients it was apparent that the administration of 50 to 200 mg of diethylstilbestrol per day did not in-

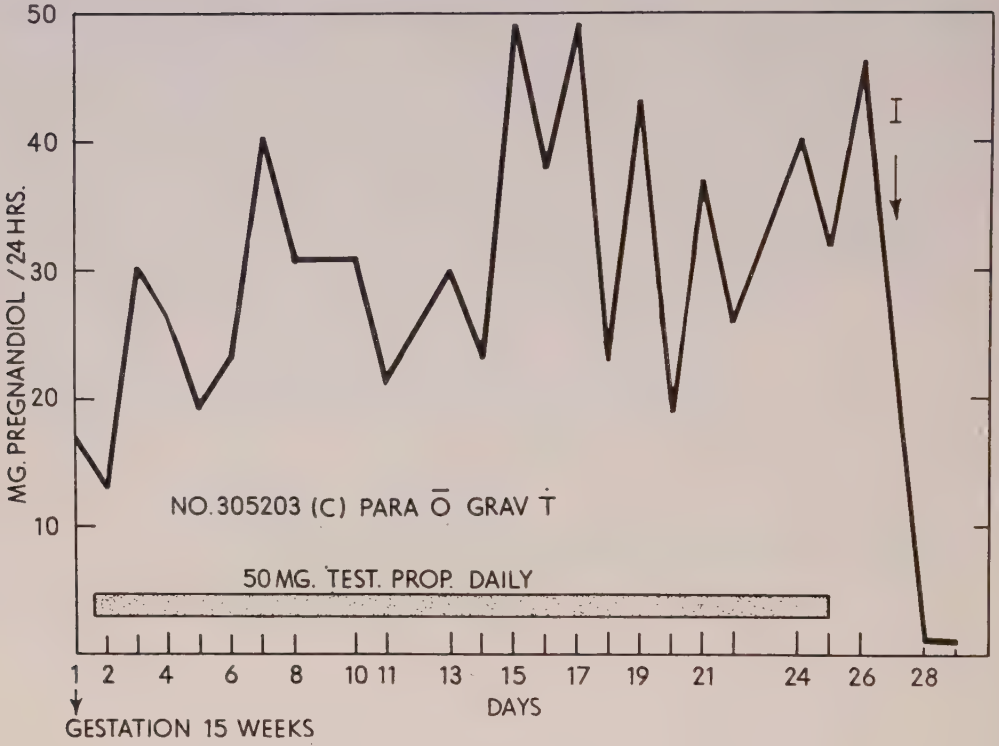

FIG. 4.
The daily intramuscular administration of 50 mg of testosterone propionate was instituted at 15 weeks gestation and continued for 25 days. This androgen did not alter the normal output of pregnandiol and the curve of excretion is typical for this period of pregnancy.

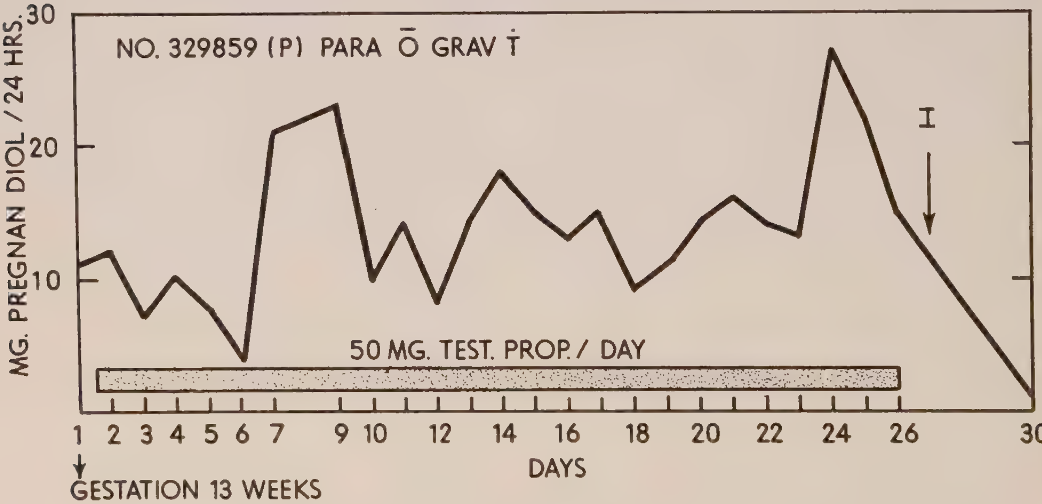

FIG. 5.
The administration of testosterone propionate was started when this patient was 13 weeks pregnant and continued for 26 days for a total of 1300 mg. No effect on pregnandiol excretion can be demonstrated.

fluence the excretion of pregnandiol in the urine. We can assume that during the first 16 weeks of the 38-week gestation this synthetic estrogen does not affect the production of progesterone by the placenta as determined by pregnandiol output. In a previ-

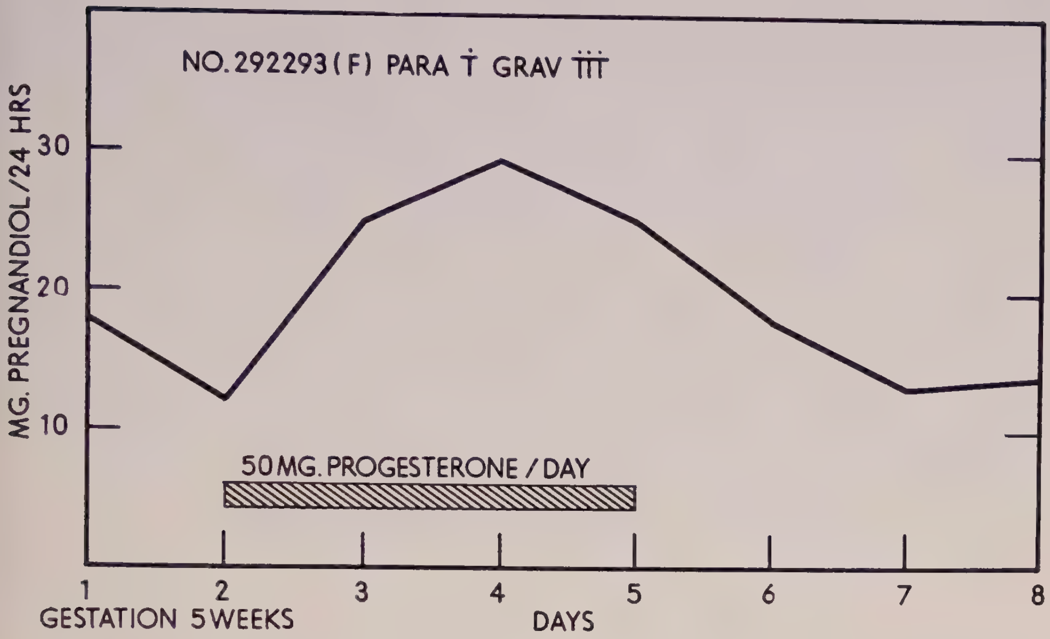

Fig. 6.

Progesterone administered at 5 weeks gestation produces a marked rise in pregnandiol excretion.

ous communication the authors[3] have suggested that diethylstilbestrol may affect the pregnancy profoundly by increasing the uteroplacental circulation thereby promoting an improvement in the uterine environment for the fetus. It is recognized that the corpus luteum is the chief source of progesterone early in pregnancy but in that period between 12 and 16 weeks a well formed placenta is present.

Testosterone proprionate. Testosterone proprionate was administered to 2 patients in order to determine if androgens could influence the metabolism of progesterone and the excretion of pregnandiol. The chemical configuration of the testosterone molecule conforms closely to that of progesterone. The first patient received 50 mg of the hormone in an oily solution by intramuscular injection each day for a period of 25 days or a total of 1250 mg. Therapy was instituted at about 15 weeks and when the fetus was delivered by abdominal hysterotomy its size and development was compatible with 18 or 19 weeks gestation. The second patient received 50 mg of testosterone proprionate each day for 26 days for a total of 1300 mg. Androgens were started at about 13 weeks gestation and the pregnancy terminated at about 17 weeks. Both of these fetuses were normal externally.

Signs of virilism began to manifest themselves when the patients received about 1000 mg of testosterone. Acne appeared on the face. A fine growth of hair could be discerned. There was some prominence of the clitoris. The most striking change was in the voice, the pitch of which dropped rapidly from high soprano to a husky masculine alto. The voice has not recovered the former range although all other findings have disappeared.

There was no obvious change in the pattern of pregnandiol excretion (Fig. 4 and 5). The usual daily variations were present but the tendency of the curve was upwards after the 15th week. At the termination of the pregnancy the pregnandiol output dropped precipitously and disappeared at the end of 72 to 96 hours. Thus, androgens like estrogens do not influence the excretion of pregnandiol in the first half of the gestation.

Progesterone. Progesterone was administered to 3 patients for varying periods at 5, 7 and 10 weeks gestation. Fifty milligrams of progesterone in oil was injected intramuscularly daily in 2 patients and in a single

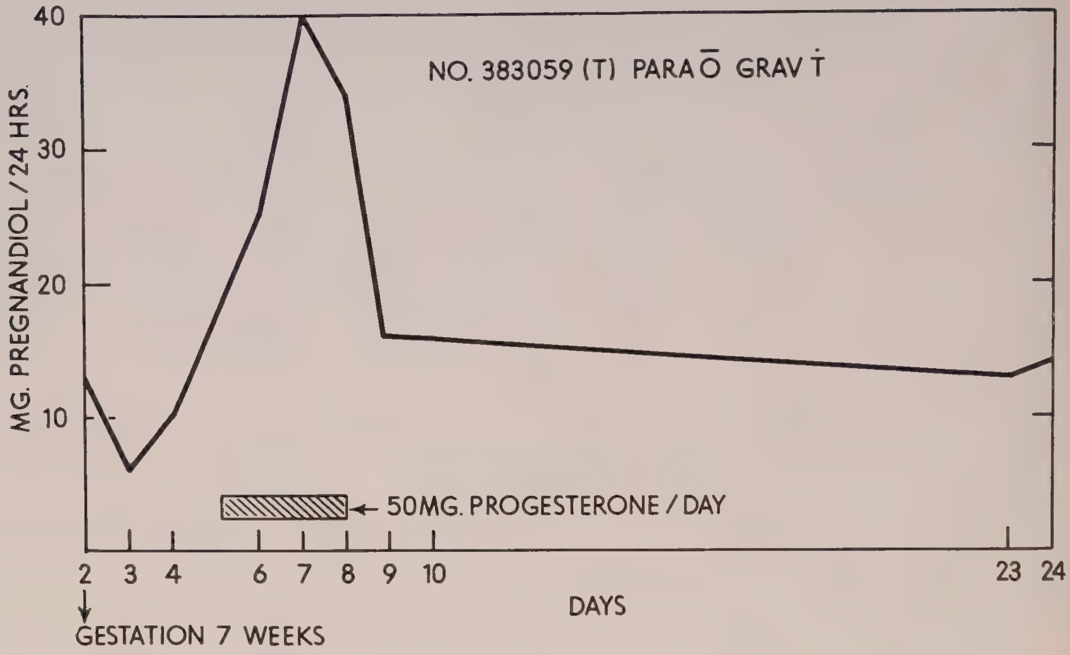

Fig. 7.
The administration of progesterone in 50 mg daily doses at 7 weeks gestation resulted in a 30 to 35% recovery of this steroid as pregnandiol.

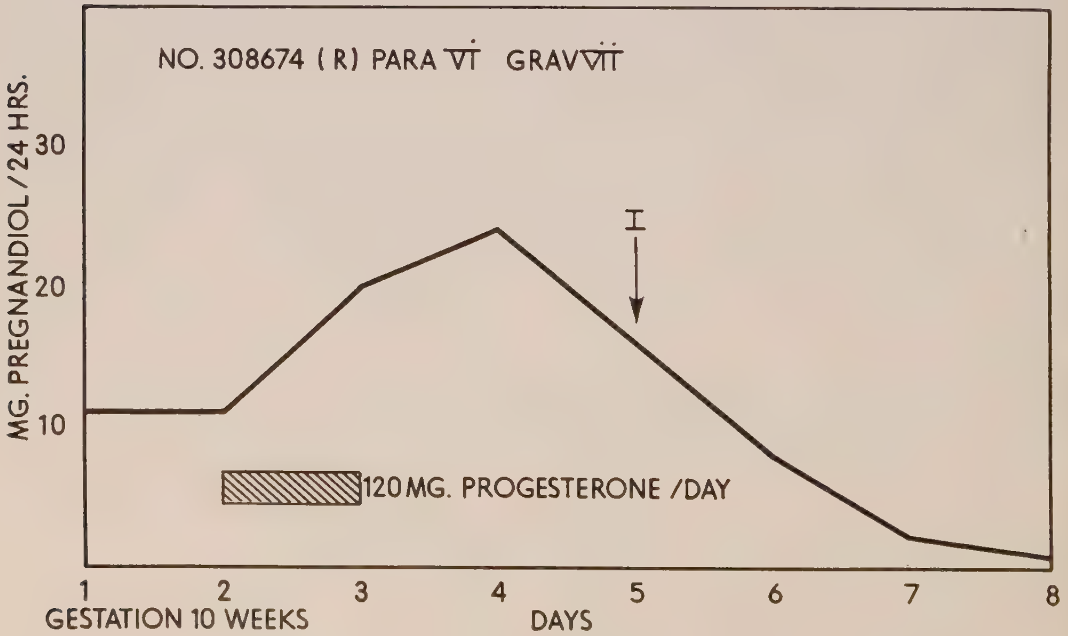

Fig. 8.
The single administration of 120 mg of progesterone to a patient at 10 weeks gestation resulted in a more prolonged increase of pregnandiol excretion but in a decrease in the total amount of the steroid recovered as pregnandiol.

dose of 120 mg in the third. In each instance there was an immediate increase in the excretion of pregnandiol. The effect of this hormone disappeared within 24 hours after the last injection (Fig. 6, 7, 8).

If the increase in pregnandiol above the average level excreted in each patient was calculated, about 30 to 35% of the progesterone injected could be accounted for as pregnandiol. Thus, in Fig. 6, 150 mg of progesterone was injected over a 3-day period and at least 50-60 mg of additional pregnandiol was recovered from the urine during this period. In the nonpregnant individual in the preovulatory phase it is possible to recover less than 10% of the progesterone as pregnandiol. The activity of the corpus luteum in the postovulatory phase and in the first trimester of pregnancy must exercise some effect on the metabolism of progesterone so that a much greater percentage can be accounted for as the inert metabolite, pregnandiol. It was also observed that a higher percentage of progesterone is recovered as pregnandiol when the steroid is administered in small divided doses than as a single large dose (Fig. 8).

Discussion. Diethylstilbestrol and testosterone propionate do not influence the excretion of pregnandiol during the first 16 weeks of normal gestation. On the contrary, progesterone is metabolized efficiently so that at least 30 or 35% is recovered as pregnandiol. If it is desirable to increase the amount of progestational hormone available during early pregnancy, it is more logical to administer progesterone in liberal amounts rather than diethylstilbestrol. However, it is the impression of the authors that there is no lack of progesterone in most of the pregnancies which threaten to end during the early months. Decreased pregnandiol levels may be an index of poor placental function.

Summary. Diethylstilbestrol, testosterone proprionate and progesterone were administered to 15 patients with normal pregnancies in order to study the effect of these substances on progesterone metabolism and pregnandiol excretion. Diethylstilbestrol and testosterone when administered daily in large amounts over long periods during the first 16 weeks of gestation did not alter the normal excretion of pregnandiol. The administration of progesterone was followed by the prompt recovery of a considerable portion of the injected steroid as pregnandiol.

We wish to thank E. R. Squibb & Company for their liberal supply of diethylstilbestrol and progesterone.

15936

A Gelatinous Variant of *Pseudomonas aeruginosa.*

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Pseudomonas aeruginosa is generally recognized as highly variable. Among the variations exhibited are those of colony form. During the course of cultural examinations made on a case of third-degree burn in 1943, a variant was isolated which seems not to have been described before. It appeared in small numbers as an unusual colonial form when the organism was streaked on a modified Sabouraud's medium. The modification of this medium consisted of the addition of a 0.5% glycerol to the usual Sabouraud's maltose-peptone agar. Subsequent observations showed that the appearance of this variant depended on the presence of the glycerol in the medium.

The initial observations were as follows: among the usual colonial forms a small number of exceptional colonies were recognized on a few of the plates. These were few enough to be sparsely scattered even in the more heavily seeded portions of the plate, but occurred here and there as well-separated colonies. They differed strikingly from the ordinary colonies and in young cultures resembled small pearls so closely that we have since referred to the variant as the "pearl form" of *Ps. aeruginosa.* Variations have been observed, however, within the form itself which make the term "pearl form" less appropriate for it than that of "gelatinous variant."

A remarkable feature of this variant, when grown on a solid medium containing glycerol, is its tendency to increase in size long after ordinary colonies have ceased to grow. If recently seeded plates are inverted and placed under a bell-jar at room temperature, growth of the individual colonies will usually continue until they have reached a diameter of a centimeter or more and a depth of several millimeters. Some eventually touch the cover of the inverted Petri dish and the more fluid of these variants often drip material onto the cover of the inverted dish. In general, however, they have a stiff jelly-like consistency and tend to preserve their form for many weeks. They become markedly elevated even when grown on upright plates. If the variant is seeded to a solid glycerinated medium by merely touching the surface of the medium at given points with an inoculating needle ("point inoculation"), exceedingly large mounds of growth are often produced. The colonies are translucent and glistening by reflected light and opaque by transmitted light. They differ in color. Smears show that virtually all of the colony consists of this gelatinous material, with relatively few organisms scattered through it. Since the organisms are much more numerous at the base of the colony, it would appear that the material is built up chiefly by the metabolic activity of organisms next to the surface of the medium. Stained preparations show that the material is acidophilic and drawn out in more or less heavy strands, many of which are stretched around perfectly clear areas, giving the mass an alveolar structure. Many of the broken strands are distinctly wavy and resemble elastic fibers in animal tissue. Although much of it occurs in strands, the general physical appearance of the material is not that of a mucoid substance.

Growth on liquid media containing glycerol is also abundant and definitely gelatinous. The jelly-like layer formed at the surface of the medium, where Pseudomonas grows best, may become more than a centimeter in thickness. The floating mass hangs together well and exhibits no stringy or mucoid properties.

We have isolated a number of variants within this gelatinous type since we first isolated the "pearl form." It is well known that ordinary strains of Pseudomonas may show many variations in colony form; also variations in pigment production. Many of the variations seen in ordinary nongelatinous colonies have counterparts in the gelatinous variant. Those first isolated were chiefly of a milky or pearly-white color; those isolated later, either as new gelatinous forms or as variants of the original pearl forms, range from amber through red to green. They differ also in form. The "pearl forms" initially isolated had evenly rounded surfaces; however, many of those isolated since from the same strain of Pseudomonas show gently sloped irregularities in their surface contours much as though the colony consisted of intertwined ropes of gelatinous growth. Some develop a long gelatinous nipple or tongue-like upgrowth from the center of the colony. There are other departures from an evenly rounded surface. These differences have been associated with basic differences in colony form observed among ordinary nongelatinous colonies of Pseudomonas. A common nongelatinous type is a low convex or pulvinate colony with a rounded surface studded or not with small papillae and with or without a thin spreading edge. Another type is relatively flat and more or less villous by virtue of interlacing strands or cords of more elevated growth drawn through the colony. This colonial form has been described in the literature as a "rugose" form.[1] Apparently the more evenly rounded "pearl form" is a gelatinous variant of the first-mentioned type of nongelatinous colony; the second a gelatinous variant of the ordinary rugose form. One may also observe among ordinary colonial forms, whether rugose or not, those which have definitely raised centers ("umbonate" colonies). The latter seem to give rise to gelatinous forms with the long nipples or tongue-like processes referred to earlier. These conclusions are based on observations which have been made on gelatinous variants seeded to glycerol-free media. When this is done the gelatinous variant reverts to the nongelatinous type. Although quite stable on glycerinated media, there is nevertheless some tendency for nongelatinous variants to arise from the gelatinous ones. This tendency becomes more evident after the variant has been grown for a time in ordinary broth and is then transferred back to a solid medium containing glycerol. Under these conditions there are usually a few colonies which are not of the gelatinous type.

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[1] Fiala, S., *Centralbl. f. Bakt. u. z. w.*, Abt. I, Orig., 1941, **148**, 58.

From the observations related above it appears that what we have here is a purely physiological variation, one involving ability to utilize glycerol for the formation of the particular gelatinous material, this functional variation not being associated with any one basic colony type. It may, in other words, be superimposed upon any one of the various colony forms known to be produced by this organism. The fact that very few colonies among the many which were originally isolated showed this peculiar physiological ability speaks for its rarity. It also seems infrequent among other strains of *Ps. aeruginosa.* So far we have been able to relate the property only to the utilization of glycerol, although the possible effects of other polyhydric alcohols are still to be investigated, likewise the nature of the gelatinous material produced.

Since a strong pyocyaneus phage was isolated from mixed broth cultures of the exudate from the lesions, it was thought possible that the variation in question might have arisen in some way from exposure to phage. The gelatinous variants, however, proved quite as susceptible as the nongelatinous forms to the phage we isolated. Moreover, filtrates of a number of broth cultures of these gelatinous variants failed to show that they themselves carry phage.

Summary. What appears to be a new variant of *Pseudomonas aeruginosa* is described. It is characterized by the production of large amounts of gelatinous material when grown on media containing glycerol, and on solid media gives rise to markedly raised colonies of a stiff jelly-like consistency and to a thick gelatinous layer near the surface of liquid media. The variation seems to be entirely one of ability to utilize glycerol for the production of the particularly gelatinous material, and to be unrelated to any one of the several basic colony forms known to occur in this species.

15937 P

Hemolytic Action of Mouse Mammary Carcinoma Filtrate on Mouse Erythrocytes *in vitro.*

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Although the association of cancer with anemia has long been observed, no satisfactory explanation of the mechanism of this relationship has yet been furnished.[1] Experiments reported in this study appear to suggest that, at least in the case of mammary carcinoma of mice, a direct destructive action of a filterable substance contained in the tumor cells, on the erythrocytes of the host, should be taken into consideration. Thus, filtrates prepared from mouse mammary carcinomas were found to destroy mouse erythrocytes *in vitro.* The following technic was employed to demonstrate this hemolytic property of the tumor extracts:

Materials and Methods. Tumor extracts. Ten different mammary carcinomas that developed spontaneously in old C3H females, and 2 transplanted tumors that originated as mammary carcinomas in C3H females and have been carried through successive transplantations in these animals,[2] were used as the source of the tumor extracts. A freshly prepared extract from a single tumor was used for each test. The tumors were removed aseptically, weighed, and ground for several minutes in a porcelain mortar, 0.85% solution of sodium chloride being added to obtain cell suspensions varying from 15 to 30%. The suspensions thus obtained were cleared by centrifugation at 5000 t.p.m. for 10 minutes, and the supernatant fluid was then passed through a Seitz (No. 3) filter; the resulting filtrate was designated "filtered extract," and was used for some of the tests. In other experiments, the tumor cell suspensions were cleared by 3 successive centrifugations at 5000 t.p.m. of 10 minutes each; the final supernatant fluid was designated "centrifugated extract" and used for the tests. Both filtered and centrifugated tumor extracts were bacteriologically sterile as evidenced by negative inoculations of ordinary culture media.

Erythrocytes. Blood was drawn directly from the heart of an etherized mouse of either the C3H[2,3] or Ak[4] inbred lines, or of the An[2] subline. The oxalated red blood corpuscles (RBC) were washed twice at 3,000 t.p.m. for 5 minutes, and a final 1% suspension of RBC in 0.85% solution of sodium chloride was prepared. The RBC suspensions were freshly prepared for each test.

Technic of the test. The entire procedure was aseptic. One cc of a 1% RBC suspension was mixed gently in a small (10 x 75 mm) test tube with 0.5 cc of either centrifugated or filtered tumor extract. Appropriate controls were made simultaneously; they consisted of RBC suspensions to which physiological saline solution, or either filtered or centrifugated extracts from normal, healthy mouse organs (liver, spleen, kidneys) prepared aseptically from mice of the An line known to be free from the mammary carcinoma agent,[2] were added. The test tubes were then placed in an incubator at 37°C for 2½ to 3 hours. After this lapse of time the tubes were removed from the incubator, centrifugated briefly at 3000 t.p.m. and the results were read.

Recording of results. The reading was made in daylight. Complete hemolysis with no sediment left was designated "three plus." Rich in color hemolysis with, however, some sediment left, was designated "two plus." Slight hemolysis was designated "one plus." The faintest trace of hemolysis above the sediment was designated "doubtful." No hemolysis at all, with a colorless supernatant fluid over a sharply demarcated sediment was designated "negative."

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[1] Taylor, A., and Pollack, M. A., *Cancer Research,* 1942, **2**, 223.

[2] Gross, L., *J. Immunol.,* 1947, **55**, 297.

[3] Strong, L. C., *Genetics,* 1935, **20**, 586.

[4] Cole, R. K., and Furth, J., *Cancer Research,* 1941, **1**, 957.

Results. Titration of the extracts. Positive results were obtained in practically all tests made with both filtrates and centrifugates obtained from tumor cell suspensions having a concentration varying from 15 to 30%. One plus results were obtained with concentrations varying from 7.5 to 15%. In most instances, either negative, or "doubtful" results could be obtained with 5 or 2.5% concentrations. Higher dilutions gave, as a rule, negative results. There were variations in the intensity of the activity of the various samples of tumor extracts made at different times, and from various tumors. Centrifugates appeared to be only slightly more potent than filtrates. Spontaneous tumors had a stronger hemolytic potency than the transplanted tumors used for the tests.

Under the conditions of our experiments, there were no positive readings among the controls consisting of tubes containing RBC to which physiological saline, or either filtered or centrifugated extracts made from normal healthy organs (liver, kidneys, spleen) removed from An mice[2] were added.

The use of various erythrocytes for the tests. The strongest hemolytic action was observed when filtrates or centrifugates prepared from tumors grown in mice of the C3H line, were mixed with erythrocytes obtained from mice of the same breed. The hemolytic action of these tumor extracts was only slightly weaker, however, for RBC prepared from mice of either the An or Ak inbred lines. In one experiment, erythrocytes were prepared from a "market" albino mouse of unknown parentage, and they were found to be also suitable for the tests. Under the conditions of our experiments, no hemolytic action of either the filtrates or centrifugates was observed when either rabbit, guinea pig, or human erythrocytes were used for the tests.

Exposure of the tumor extracts to 56°C. The hemolytic action of the tumor extracts was found to be only slightly, if at all, inhibited, following exposure of the extracts, prior to tests, to 56°C in a waterbath for 30 minutes. Brief boiling destroyed, however, the hemolytic potency of the tumor extracts.

Hemolytic action of mouse leukemia filtrates on mouse RBC in vitro. Filtrates prepared from either spontaneous, or transplanted lymphatic leukemia of Ak mice[4] were found to possess hemolytic action, similar to that of the mammary carcinoma extracts, *in vitro,* on red blood corpuscles obtained from mice of either the Ak, C3H, or An inbred lines. This action was strongest on erythrocytes prepared from the blood of Ak mice. No hemolytic action occurred when human, rabbit, or guinea pig RBC suspensions were used.

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15938 P

Streptomycin II, An Antibiotic Substance Produced by a New Species of Streptomyces.*†

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The production of streptomycin by actinomycetes is limited to certain strains of a single species of the genus *Streptomyces,* known as *S. griseus.* In addition to the first 2 streptomycin-producing strains isolated in 1943,[1] only 3 other streptomycin-producing strains have thus far been reported. Although over a hundred cultures of *S. griseus,* isolated from different substrates, have now been tested in our laboratory for their antibiotic properties, only one culture was found[2] capable of producing streptomycin; several others were capable of producing antibiotic substances such as grisein,[3] which differed, however, from streptomycin. Similar results were obtained by Carvajal,[4] who isolated 2 streptomycin-producing strains of *S. griseus.* No other organisms culturally different from *S. griseus* have been found capable of producing streptomycin.

In connection with a study of the bacterial population of the waters around Bikini and Rongelap Atolls during the recent atomic bomb experiments in the Marshall Islands,‡ a culture of an actinomycete was isolated from one of the Bikini soils. This culture showed strong inhibition of various Gram-positive and Gram-negative bacteria, as well as of acid-fast bacteria. This culture belonged to the genus *Streptomyces,* but was distinct from streptomycin-producing *S. griseus.*

This culture was very similar, both morphologically and culturally, to *S. griseolus* (Actinomyces 96) described by one of us in 1919.[5] It differed, however, in some of its cultural properties and in its ability to produce an active antibiotic substance. Because of these differences and also because of the peculiar natural substrate from which this culture was isolated, it is proposed to designate it as *Streptomyces bikiniensis.* It produces on synthetic and on organic media containing glucose, a dark gray aerial mycelium, which consists of dichotomous branches with straight chains of conidia. A thick complete pellicle is formed on the surface of stationary cultures, with protein-containing media being pigmented brown to black.

Cross streak tests of the culture were made using various test bacteria. The zones of inhibition compared favorably both with streptomycin-producing strains of *S. griseus* and the streptothricin-producing *S. lavendulae.*

The highest antibiotic potency was produced by the organism both under stationary and submerged conditions of culture on media commonly used[1] for the production of streptomycin. The antibiotic spectrum of the crude culture filtrate suggested a definite similarity of this substance to streptomycin. This similarity was confirmed when the active substance was isolated from the culture medium. The titre of the crude culture filtrate ranged in activity from 200 to 250 streptomycin units per ml. The corresponding activity against the various bacteria, such as *Escherichia coli, Bacillus subtilis, Bacillus mycoides, Staphylococcus aureus* and *Myco-*

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* Journal Series Paper, New Jersey Agricultural Experiment Station, Rutgers University, Department of Microbiology.

† Partly supported by a grant made by the New York Foundation.

[1] Schatz, A., Bugie, E., and Waksman, S. A., Proc. Soc. Exp. Biol. and Med., 1944, **55**, 66.

[2] Waksman, S. A., Reilly, H. C., and Johnstone, D. B., *J. Bact.,* 1946, **52**, 393.

[3] Reynolds, D. M., Schatz, A., and Waksman, S. A., Proc. Soc. Exp. Biol. and Med., 1947, **64**, 50.

[4] Carvajal, F., *Mycologia,* 1946, **38**, 596.

‡ This work was done during the Operation Crossroads, in 1946, where the senior author was a civilian biological investigator.

[5] Waksman, S. A., *Soil Sci.,* 1919, **8**, 121.

bacterium tuberculosis, was comparable to that of streptomycin.

Isolation of the active substance has been obtained by methods used to isolate streptomycin. The methyl alcohol formic acid procedure yielded preparations assaying 158 streptomycin units per mg.

The crude preparation thus isolated from the culture filtrate of *S. bikiniensis* had no activity against fungi. Bacteria made resistant to streptomycin were also resistant to this substance. It was inactivated by cysteine, it gave reduced activity in the presence of glucose, and it was sensitive to increased acidity to the same degree as streptomycin. These results tend to establish the fact that the new antibiotic is similar to streptomycin if not identical with it.

The toxicity of the preparation was determined by the use of chick embryos. As much as 600 units of the material, deposited upon the chorio-allantoic membrane, and concentrations greater than 1200 units injected into the yolk sac, of 11-day embryos gave 100% survival. One may thus conclude that the minimum lethal dose of the new antibiotic is relatively high, at least in the chick embryo.

As long as this preparation has not been crystallized and as long as its activity *in vivo* against various bacteria has not been determined, it is not possible to state definitely that the new antibiotic is streptomycin. Some question may also be raised concerning its absolute identity with streptomycin, since it is produced by a totally different organism and occasional quantitative differences in the degree of sensitivity of certain bacteria to this antibiotic as compared with streptomycin are obtained. For these reasons, it is proposed to designate the new preparation as Streptomycin II.

15939 P

Preparation of Hemoglobin Solutions Containing Hemoglobin Reducing Enzymes.

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Employing the following procedure we are able consistently to obtain sterile solutions, free from cellular debris, containing enzymes which bring about the consumption of the entire oxygen content of the solution, thereby converting the hemoglobin present into the extremely stable reduced form.

Procedure. Human erythrocytes are separated from the plasma by means of a bucket centrifuge. These cells are suspended in 2 volumes of a solution containing 6% glucose, 0.15% nicotinic acid amide and 0.0006% ammonia. This suspension is centrifuged at approximately 20,000 r.p.m. in a Sharples separator-type bowl that separates the cells from the wash solution and at the same time lyses them, though with little loss of hemoglobin into the wash solution. The washed, lysed cells, having a hemoglobin concentration of approximately 30%, are delivered directly into 100 cc of distilled water containing sufficient nicotinic acid amide to produce a concentration of 0.15% in the final product, which is diluted to contain approximately 7% hemoglobin. The mixture is diluted with 2 volumes of distilled water containing sufficient glucose to produce an estimated final glucose concentration of 6%. The diluted mixture is adjusted to pH 5.8 with N/10 HCl and is centrifuged through a Sharples clarifier-type bowl to remove the stromata. The centrifugate is stirred for 30 minutes with "Decalso," 30 g per liter, from which it is decanted. An amount of normal NaOH solution calculated to neutralize the HCl is added. These last 3 steps have been adopted from the work of Hamilton and Farr.[1] Ammonium hydroxide is added in the amount of 0.6 mg of NH_3 per 100 cc. Clarifying and sterilizing filtrations are carried out using Republic K6 and S6 pads. The entire procedure is carried out at 0°C in the course of 8 hours.

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[1] Hamilton, P. B., and Farr, L. E., *Fed. Proc.*, 1946, **5**, 136.

Solution 1029-D3 is an example of solutions produced as outlined above. Its behavior during storage was compared (Table I) with that of a solution in the preparation of which the use of ammonia was omitted (Solution 1029-D6). Other modifications of the procedure (elimination of nicotinic acid amide, elimination of glucose, use of isotonic saline, etc.) gave results closely resembling those of 1029-D6.

The Evelyn photoelectric colorimeter was used for the measurement of total hemoglobin and methemoglobin, as described by Evelyn and Malloy.[2] The oxygen content of the solutions was measured by the technic of Van Slyke and Neill.[3]

At the end of 15 days at room temperature, or 7 days at 37°C, Solution 1029-D3 had assumed the "grape-juice" color of reduced hemoglobin, which color was retained for months. During the storage experiments, 1029-D6 became progressively browner, until after 39 days at room temperature and 4 days at 37°C the brown methemoglobin almost completely masked the brilliant red of the remaining oxyhemoglobin.

Summary. Hemoglobin solutions were prepared from erythrocytes that were laked in the presence of nicotinic acid amide, glucose, and ammonia. The solutions were Seitz-filtered, yielding clear solutions free from cellular debris, and containing sufficient of the enzyme systems of the cell to change the pigment from oxyhemoglobin to reduced hemoglobin with no appreciable accumulation of methemoglobin during the process. The solutions could be stored in the reduced state for protracted periods at elevated temperatures without increase in methemoglobin content.

TABLE I.

Sol'n No.	Total Hb, %	% of total Hb. appearing as MetHb. after storage at room temp. for period indicated									% oxyhemoglobin of the solutions stored at 37°C for period indicated					
		Initial	2 days	3 days	6 days	7 days	15 days	16 days	39 days	40 days	Initial	1 day	2 days	3 days	4 days	11 days
1029-D3	7.36	0.52	1.3			1.89	0.55			1.5	7.79	6.57	4.55		1.59	0
1029-D6	6.36	3.08		15.62	18.3			38	73.2		6.12	1.59		0.46	0	

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[2] Evelyn, K. A., and Malloy, H. T., *J. Biol. Chem.*, 1938, **126**, 655.

[3] Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924, **61**, 523.

15940 P

Intra-Arterial Fluorescein for Evaluation of Peripheral Vascular Diseases.

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Lange and Boyd[1,2] introduced the use of fluorescein as an aid in the diagnosis and in the estimation of prognosis of peripheral vascular diseases. They injected 5 to 10 cc of a 5% solution of sodium fluorescein intravenously after making a series of superficial scratches from the base of the toes to the midthigh, and then examined the affected leg under an ultraviolet lamp. Because ultraviolet light penetrates only 2 to 3 mm, scratches enable the examiner to evaluate more accurately the existence of fluorescence.

Neller[3,4] employed wheal fluorescence, both scratch and histamine, in conjunction with the fluorescein technic, and stated that the wheal was the only phase of the tissue response to histamine or injury which reflected accurately the local blood flow. He favored the fluorescent wheal scratch technic for routine clinical use.

At this clinic we had employed these technics but were disappointed in the low intensity of fluorescence and related this to the dilution of the intravenously-injected dye by the total blood volume. Interpretation of the vascular status following intravenous fluorescein appeared but little more objective than the McClure-Aldrich and other similar tests.

In an attempt to secure a more clear-cut definition of circulatory inadequacy, 5 cc of a 20% solution of sodium fluorescein was injected intra-arterially. Following injection of the dye, fluorescence of the entire extremity was apparent in a few seconds. The intensity of fluorescence was many times greater than that obtained when the dye was injected intravenously. In areas of impending gangrene there was a much sharper line of demarcation. Thus, the test became more objective, and was readily adaptable to quantitative fluorometric measurement.

With the intra-arterial method, the value of the previously described scratch technic was enhanced, permitting more clear-cut definition of the extent of impairment of arterial inflow to the extremity. The often sharply localized vascular injury seen in cases of presenile gangrene accompanying diabetes mellitus was in marked contrast to the generalized mottled appearance revealed in cases of diffuse arteriosclerotic disease.

In addition, after intra-arterial injection, the dye is more slowly released into the general circulation, and hence unpleasant side effects such as transitory nausea appear less frequently.

Summary. Intra-arterial administration of fluorescein offers an objective approach to the impaired arterial inflow to the extremities. The method is adaptable to quantitative interpretation, and minimizes the number of unpleasant side reactions.

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15941

Report of Failure of Alpha-tocopherol to Prevent Diethylstilbestrol-Induced Purpura in Dogs.*

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Castrodale *et al.*[1] showed that diethylstilbestrol given in large doses parenterally to dogs produced thrombopenia in about 8-14 days and hemorrhagic manifestations several days thereafter. This was accompanied by changes in the peripheral blood, the bone marrow, and in some instances, the liver prior to death. Tyslowitz and Dingemanse,[2] and Crafts[3] described similar results in dogs with the former investigators reporting the development of an aplastic anemia after a latent period of 1-3 weeks. Liver extract injections did not prevent the development of the anemia. Skelton, Shute *et al.*[4] reported that the administration of synthetic alpha-tocopherol acetate (Ephynal Acetate — Hoffman-La-Roche) in oral doses of 200 mg per day corrected the diethylstilbestrol-induced purpura of dogs and caused the platelet counts and capillary fragility to return to normal. If employed earlier, the appearance of frank purpura and blood vascular deficiency was prevented. The study here reported was made in an attempt to confirm the observations of Skelton and his associates.

Methods. Three male and 3 female adult dogs were used as experimental animals. The dogs were observed over a period of 5-11 weeks. The diet consisted of Dickinson Dog Food without additional supplement. The following determinations were done twice weekly throughout the experiment: capillary fragility test, bleeding time, coagulation time, and platelet count. In addition, erythrocyte and leukocyte counts, hemoglobin determinations, and differential counts were done at weekly intervals on Dogs 4, 5, and 6 throughout the investigation, and on Dogs 1, 2, and 3 during the latter part of the investigation. Ten cc blood samples were drawn from the femoral arteries into syringes coated with liquid paraffin. The blood samples were then placed in small paraffined beakers containing 0.5 cc of 2.5% sodium citrate. All blood determinations other than differential counts were done from these specimens. Capillary fragility tests were done by a method modified from Dalldorf[5] which employed a negative pressure of 180 mm mercury for one minute over a 4 cm diameter circular area of the abdominal wall. Bleeding times were done by the Duke method. Coagulation times were determined by the 2-tube method of Lee and White in a 37-degree water bath. Platelets were counted by the direct wet method using Rees-Ecker diluting fluid. The red counts were made using the same diluting fluid. Differential counts were obtained from Wright stained smears of arterial blood. Hemoglobin values were determined by the Haden-Hausser method; hematocrit readings, by the Wintrobe method.

In addition, bone marrow biopsies were taken from the ribs of Dogs 4, 5, and 6 at the beginning and termination of the experiment. In Dogs 1, 2, and 3 biopsies were made only at the end of the experiment. Two or 3 control determinations were made on each dog immediately prior to experimental study.

Four dogs received daily intramuscular injections of approximately 1.5 mg per kg body

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[1] Castrodale, D., Bierbaum, O., Helwig, E. B., and MacBryde, C. M., *Endocrinology*, 1941, **29**, 363.

[2] Tyslowitz, R., and Dingemanse, E., *Endocrinology*, 1941, **29**, 817.

[3] Crafts, R. C., *Endocrinology*, 1941, **29**, 606.

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[5] Dalldorf, Gilbert, *J. Exp. Med.*, 1931, **53:2**, 289.

TABLE I.
Effects of Diethylstilbestrol and Diethylstilbestrol Plus Alpha-tocopherol in Dogs.

Dog	mg/K	Day	Pet.	B.T.	C.T.	Plat.	Hb.	RBC	WBC
1 ♀	1.5 diethylstilbestrol	C	0	2.5	6	228			
		18	3	15.<	18	12			
		18	Alpha-tocopherol started 15 mg/K.						
		25	8	15.<	24	18			
		39	0	15.<	30	20	5.25	2.13	
		40	Diethylstilbestrol stopped.						
		44	1	15.<	26	24	3.7	1.23	2.2
		51	Dog expired. Autopsy performed.						
2 ♀	1.5 "	C	1	2.	12	256			
		18	11	19.<	18	24			
		18	Diethylstilbestrol stopped. Alpha-tocopherol started 15 mg/K						
		25	15	15.<	38	20			
		50	0	5.5	12	186	12.5	5.47	9.95
		52	Diethylstilbestrol started 1.5 mg/K.						
		69	0	1.5	15	38	12.0	5.51	38.45
3 ♀	1.0 "	C	0	2.	8	292			
		22	0	5.5	21	44			
		40	0	2.	16	218			
		42	Diethylstilbestrol dosage increased to 2 mg/K.						
		64	0	1.	13	180	9.0	3.88	27.95
		71	0	1.5	11	266	9.5	4.07	35.30
4 ♂	1.5 "	C	1	3.	11	266	13.5	5.62	14.25
		13	0	1.	11	58	12.5	5.93	31.25
		20	0	1.	28	22	12.5	4.80	49.50
		34	0	1.	18	172	10.5	4.70	20.75
		41	0	2.5	20	206	11.	5.39	16.85
5 ♂	1.5 " 15 alpha-tocopherol	C	1	0.5	13	230	12.0	5.27	15.75
		13	0	3.	14	38	10.5	5.39	36.20
		20	0	15.<	41	18	10.	4.91	112.30
		34	0	6.	20	82	8.	3.85	15.60
		41	0	1.	17	94	10.	4.30	10.45
6 ♂	1.0 diethylstilbestrol	C	2	1.	16	196	14.0	6.67	12.6
		17	0	1.	6	10	13.5	6.17	31.2
		24	Diethylstilbestrol stopped.						
		25	6	15.<	23	18	8.5	3.04	55.
		32	Dog expired. Autopsy performed.						

Days numbered from initiation of medication as listed under column "Dog."
C = Control.
Pet = Number of petechiae in 4 cm diameter circle.
B.T. = Bleeding time in minutes.
C.T. = Coagulation time in minutes.
Plat. = Platelets in thousands per cmm blood.
Hb = Hemoglobin in g per 100 cc blood.
RBC = Erythrocyte count in millions per cmm blood.
WBC = Leukocyte count in thousands per cmm blood.

weight, while 2 dogs (No. 3 and 6) received approximately 1 mg per kg of diethylstilbestrol (Eli Lilly & Co.)

Alpha-tocopherol was employed in 3 dogs in a daily oral dose of approximately 15 mg per kg body weight. In Dogs 1 and 2 it was employed 18 days after the initiation of diethylstilbestrol when hemorrhagic manifestations were apparent. In Dog 5 and later in Dog 2 it was employed simultaneously with diethylstilbestrol.

Results. The changes following diethylstilbestrol administration paralleled those previously described by Castrodale,[1] Tyslowitz,[2] and Crafts.[3] There was a marked fall in platelets reaching the lowest level in 15-20 days. Associated with the severe thrombopenia there was a lengthening of the bleeding time in all dogs (less pronounced in Dogs 3 and 4) and an increased capillary

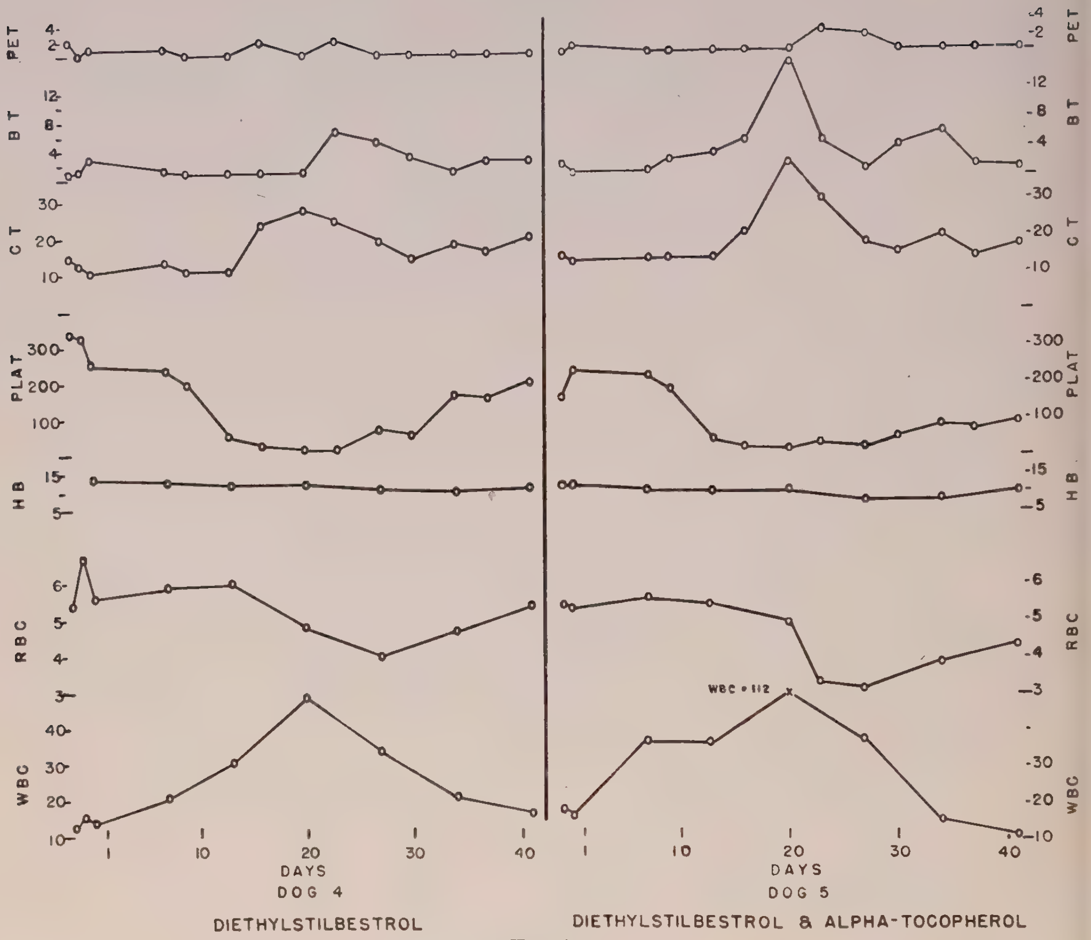

FIG. 1.

Comparative Effects of Diethylstilbestrol Plus Alpha-tocopherol and Diethylstilbestrol in Dogs, Demonstrating Similarity in Nearly All Aspects.

Days numbered from initiation of medication as listed under Table I.
Pet. = Number of petechiae in 4 cm diameter circle.
B.T. = Bleeding time in minutes.
C.T. = Coagulation time in minutes.
Plat. = Platelets in thousands per cmm blood.
Hb. = Hemoglobin in g per 100 cc blood.
RBC = Erythrocyte count in millions per cmm blood.
WBC = Leukocyte count in thousands per cmm blood.

fragility (relatively insignificant in Dogs 3 and 4) which appeared 2-10 days after the fall in platelets. All dogs in which blood counts were done showed a leukocytosis reaching its height about the 20th day and slowly regressing thereafter. In one dog the count reached 113,300 cells per cmm on the 20th day. Concomitantly there was a gradual reduction in the number of erythrocytes and the amount of hemoglobin. It is to be noted that diethylstilbestrol likewise produced a prolongation of the coagulation time with a maximum effect after 20-25 days. To our knowledge there have been no previous reports concerning the effect of diethylstilbestrol on the blood coagulation time.

In 2 dogs (3 and 4) the platelet count, bleeding time and all the blood elements returned to normal despite the continued administration of diethylstilbestrol. The simultaneous administration of alpha-tocopherol did not prevent the occurrence of thrombopenia in Dogs 2 and 5 nor did it prevent the appearance of nor modify the severity of purpuric manifestations in Dog 5. In Dog 1 in which there was a fatal outcome, little if any beneficial effect upon the marrow was noted from the use of alpha-tocopherol; while the favorable course of Dog 2 may well have been due to the early discontinuance of diethylstilbestrol. In no instance did alpha-tocopherol tend to delay the appearance of the hemorrhagic tendency nor accelerate the return to normal above that noted in Dogs 3 and 4.

Dogs receiving alpha-tocopherol and diethylstilbestrol showed changes in the erythrocyte count, leukocyte count, hemoglobin values, and hematocrit determinations similar to those receiving diethylstilbestrol alone. Leukocytoses similar to that observed in Dog 4 have been described previously by Castrodale.[1] In the 2 dogs in which death occurred (Dogs 1 and 6) postmortem examinations were done within 12-18 hours and both showed evidence of severe anemia, numerous hemorrhages throughout the body, and liver necrosis which was extensive in Dog 6. There was a marked hypoplasia of all elements of the bone marrow in Dog 1. Rib biopsies performed at the start of the investigations on Dogs 4, 5, and 6 showed normal marrow. Marrow studies postmortem or by biopsy at the conclusion of the studies showed: a markedly hypoplastic marrow in one (Dog 1), hyperplastic marrow in 2 (Dogs 3 and 4), and normal marrow in 3 (Dogs 2, 5, and 6). The type of marrow found appeared to have no significant relationship to the dosage of diethylstilbestrol nor to the administration or nonadministration of alpha-tocopherol.

Summary. Diethylstilbestrol caused a prolongation of the bleeding and coagulation time in dogs, thrombopenia, an increase in the capillary fragility, leukocytosis and a gradual anemia. In 2 dogs death resulted. The changes in the circulating blood and bone marrow produced by diethylstilbestrol appear to be temporary in some instances. The return to normal, despite the continued administration of the drug, might indicate that a tolerance to diethylstilbestrol had been acquired. This return to normal was as prompt and as great without as with the administration of alpha-tocopherol.

The present studies failed to confirm the report of Skelton, Shute, *et al.* that alpha-tocopherol exerts an antipurpuric action on the hemorrhagic diathesis induced in dogs by diethylstilbestrol.

We are indebted to Hoffmann-LaRoche, Inc., for supplying the ephynal acetate and to Eli Lilly & Co. for supplying the diethylstilbestrol.

15942

Failure of Adrenal Cortical Activity to Influence Circulating Antibodies and Gamma Globulin.*

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Studies attempting to demonstrate a relationship between adrenal cortical activity and circulating antibodies have yielded con-

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tradictory results.[1] Serum antibody levels in adrenalectomized animals have been reported to be unchanged,[2] elevated[3,4] and diminished.[5] Recently, however, Chase, White and Dougherty[6] have suggested that gamma globulin and antibodies are released from lymphocytes under the influence of the adrenal cortical hormone. This hypothesis is based upon the observation that the injection of adrenal cortical extract (ACE) brings about the "dissolution" of fixed and circulating lymphocytes[7,8] and, concomitantly, causes increased amounts of gamma globulin[9] and antibody[10,11] to appear in the blood. It was also reported that repeated simultaneous administration of ACE and antigen to intact animals produces higher antibody titers than does injection of antigen alone.[6]

The differences in antibody titers in the latter experiments,[6] though persistently obtained in several species, were rather small, since the values reported were agglutination endpoint titers, which are generally considered subject to error by a factor of 2. Furthermore, the changes produced by injection of ACE into an intact animal are probably the result of several variables and should not be attributed exclusively to direct action by the extract. It is well known that the administration of ACE produces adrenal atrophy. Since the extract does not completely replace all adrenal cortical functions there may result an indeterminate deficit of some functions and perhaps an excess of others. The findings in such an experiment,[6] therefore, reflect not only the effect of ACE, but possibly also a complex dysfunction of the animals' own adrenals.

In the present study an attempt was made to avoid the above difficulties by employing quantitative methods for the measurement of antibodies, and by studying the effect of ACE in adrenalectomized animals maintained on sodium chloride and desoxycorticosterone acetate (DCA). The latter (DCA) has been found to have no demonstrable effect on serum antibody levels.[10] The objectives of the experiments described below were to determine, firstly, whether adrenal cortical activity is essential for the formation and release of antibodies, and secondly, whether ACE is effective in enhancing circulating antibody levels in the absence of the adrenal cortex.

Experimental. Twenty-seven male albino rats, ranging in age from 14 to 18 weeks, and in weight from 215 to 252 g, were subjected to bilateral adrenalectomy under ether anesthesia. Following the operation, and for the duration of the experiment (30 days), all animals were maintained on Rockland rat diet, and on drinking water containing 1% NaCl. Each animal also received a subcutaneous injection of 0.4 mg DCA‖ every second day. Thirteen of these animals (Group I) were given, in addition, a daily subcutaneous injection of 0.5 ml of adrenal cortical lipoextract (ACE).¶ The remaining 14 animals served as controls (Group II). Six unoperated male albino rats of similar age and weight were included as additional controls (Group III). The 3 groups were composed of littermates.

All animals were immunized with a formalinized pneumococcus type IS (Dawson M Strain) vaccine (20 μg N per ml). Following an initial intraperitoneal injection

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‖ Generously supplied by Schering Corp., Bloomfield, N.J.

¶ Upjohn Company, Kalamazoo, Michigan.

TABLE I.
Comparison of Body Weights and Weights of Thymus and Pituitary Glands.

Group	Procedure	No. animals	Initial body wt, g	Final body wt, g	Thymus wt, (±Σm) mg	Pituitary wt, (±Σm) mg
I	Adrenalectomized 0.4 mg DCA 0.5 cc ACE	13	283	310	230 ± 12.5	13.2 ± 0.5
II	Adrenalectomized 0.4 mg DCA	11	267	296	318 ± 16.7	10.8 ± 0.4
III	Non-operated controls	6	272	302	226 ± 10.7	10.1 ± 0.4

of 0.5 ml on the 8th day after operation, each animal received 3 weekly courses of 4 intravenous injections each. The daily dose in the first course was 0.5 ml, in the second course 0.8 ml (average), and in the third course 0.5 ml, a total of 160 μg of bacterial N per animal. Occasionally a subcutaneous or intraperitoneal injection was given in lieu of an intravenous one. Such substitution occurred in 1.9%, 9.6% and 2.8% of the injections for Groups I, II and III respectively.

Beginning with the second course of pneumococcus vaccine each, but one, of the remaining 8 pneumococcus injections was accompanied by a simultaneous injection of 0.5 ml of a washed sheep erythrocyte suspension, increasing in strength from 2% to 4%. Five of these injections were given intravenously in admixture with the pneumococcus vaccine, and 2 were given separately by the peritoneal route. Five days after the last injection of antigen the animals were bled from the heart and autopsied. Hearts, kidneys, thymus and pituitary glands were weighed and prepared for histological examination.

Two-fold serial dilutions of inactivated sera from individual animals were tested for agglutination and hemolysis of washed sheep erythrocytes. Two 100% units of guinea pig complement were used in the latter determinations. The endpoints adopted were 3+ for agglutinins (large clumps) and 2+ for hemolysins (approximately 50% lysis).

Precipitins against Type I Pneumococcus capsular polysaccharide were determined on individual sera by the quantitative micromethod of Heidelberger and MacPherson.[12] Since a preliminary test showed that in the rat the complement present did not measurably contribute to specific precipitate N its removal as recommended for human serum by the above authors was omitted. The factor for conversion of the optical density of the blue-green color given by the Folin phenol reagent to micrograms of N was determined on electrophoretically separated rat serum gamma globulin and found to be 108 (Coleman Model II Spectrophotometer, 13 mm cuvettes, 650 mμ wavelength).

Sera of individual animals and pooled samples were studied electrophoretically after diluting 1:3 with, and dialyzing against, a pH 7.4 buffer 0.02 M with respect to sodium phosphate and 0.15 M with respect to sodium chloride.

Results. The animals survived the postoperative period in apparently good condition. All 3 groups gained weight at an identical rate during the 30 days of experimentation (Table I), but 3 animals in Group II died. The cause of the deaths was not apparent, but previous experience has shown that injections of pneumococcus vaccine may kill a small percentage of even normal animals. At autopsy none of the adrenalectomized animals had remnants of adrenal tissue demonstrable grossly.

The data on thymus weights, summarized in Table I, show that the thymus glands of the intact controls (Group III) and of the adrenalectomized animals receiving ACE (Group I) were identical in weight. This indicates adequate replacement of the adrenal factor responsible in normal animals for the reduction of lymphoid tissue in response to such stimuli as the injection of vaccine. The thymus weights of the adrenalectomized animals receiving DCA alone (Group II) were

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[12] Heidelberger, M., and MacPherson, C. F. C., *Science*, 1943, **97**, 405.

TABLE II.
Comparison of Serum Antibody Concentrations.

Group	Procedure	No. animals	Agglutinin titers		Hemolysin titers		Precipitins (SI) ±Σm μg N per cc
			Avg	Range	Avg	Range	
I	Adrenalectomized 0.4 mg DCA 0.5 cc ACE	13	1:80	1:16-1:256	1:86	0-1:160	32.1 ± 3.7
II	Adrenalectomized 0.4 mg DCA	11	1:67	1:32-1:128	1:160	1:10-1:320	35.6 ± 3.5
III	Non-operated controls	6	1:56	1:32-1:128	1:85	1:10-1:320	45.3 ± 3.7

TABLE III.
Electrophoretic Fractionation of Serum Proteins.

No. of serum samples used	Composition %				Total area of pattern (arbitrary units)
	Albumin	α-globulin	β-globulin	γ-globulin	
	Group I. Adrenalectomized, DCA + ACE.				
1	69.4	—	13.8	16.8	1505
3	60.4	8.8	9.6	21.2	1465
4	66.0	—	13.5	20.5	1485
1	67.7	—	14.6	17.7	1300
Weighted avg	64.7	2.9	12.9	20.0	1460
	Group II. Adrenalectomized, DCA.				
1	60.6	9.5	10.2	19.7	1270
1	68.8	—	13.8	17.4	1380
3	66.0	—	13.8	20.2	1235
Weighted avg	65.5	2.0	13.0	19.5	1270
	Group III. Non-operated Controls.				
1	59.0	7.4	8.1	25.5	1490
1	63.4	—	14.7	21.9	1230
Avg	61.2	3.7	11.4	23.7	1360

significantly higher than those of the other 2 groups. This difference, however, does not indicate thymus hyperplasia in Group II, since the weights of these glands were still somewhat below the accepted norm.[13,14] Despite the difference in thymus weights between the adrenalectomized animals receiving ACE and those which did not, there was no statistically significant difference between their concentrations of circulating antibodies (Table II). This was true of hemoagglutinins and hemolysins as well as of antipneumococcus capsular polysaccharide precipitins which were measured quantitatively. The unoperated control animals had slightly greater amounts of circulating precipitin than both groups of adrenalectomized animals (Table II). Statistically, this difference is probably significant.

The failure of ACE to influence the level of circulating antibodies in adrenalectomized rats was paralleled by an inability to alter the concentration of serum gamma globulin, as is indicated by the data of Table III and as is illustrated in the typical patterns of Fig. 1.

Although not bearing directly on the question of antibody formation, the following additional data were obtained. Cardiac and renal enlargement have been observed in intact animals following the repeated injection

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[13] Stoerk, H. C., *Endocrinology*, 1944, **34**, 329.

[14] Stoerk, H. C., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 90.

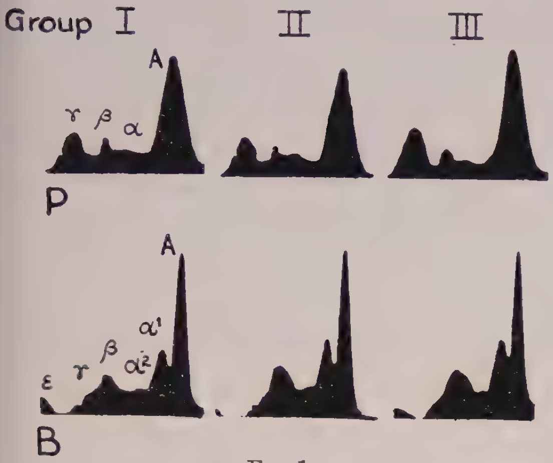

FIG. 1.
Electrophoresis patterns of sera from the 3 groups of rats. Upper row, in phosphate buffer; lower row, in barbiturate buffer.

of 10 mg,[15] and 2.5 mg[16] of DCA. The repeated injection of 0.4 mg DCA into adrenalectomized animals produced no such effect: The weights of the hearts and of the kidneys of both groups of adrenalectomized animals (I and II) were alike and were within normal limits.[17] The histological changes observed in kidneys of intact animals repeatedly injected with 2.5 mg DCA[16,18] were also present in both groups of adrenalectomized animals (receiving 0.4 mg), although to a milder degree.

The ACE-injected adrenalectomized animals (Group I) had pituitary glands which were significantly heavier (Table I) than those of the 2 control groups (II and III). This to our knowledge has not been previously observed. No apparent changes were evident, however, in the cytology of the enlarged hypophyses.

Discussion. Although the above findings indicated that adrenal cortical activity does not influence the concentration of circulating antibodies and gamma globulin, they did not exclude the possibility that considerable differences in their "turnover" had been induced: Thus ACE-injected animals may have had an augmented rate of antibody release which was obscured by an equally increased rate of antibody degradation. Conversely the adrenalectomized rats, not given ACE, may have had a diminished release of antibody which was concealed by an equally reduced rate of antibody degradation. Such changes could have occurred in the 2 groups of adrenalectomized rats, despite their having identical serum antibody and gamma globulin concentrations. This possibility had to be seriously considered in view of the widely held belief that protein catabolism is accelerated by increased adrenal cortical activity. Furthermore, it has been suggested that the metabolism of antibodies and of other serum proteins and of organ proteins are alike.[19,20] Consequently, 3 groups of rats maintained under the above experimental conditions were fed glycine tagged with heavy nitrogen and the "turnover" of their serum proteins was determined by following the rate of replacement of N^{15} by N^{14} in their total serum proteins. The results of these experiments will be reported elsewhere in greater detail.[21] It was found, however, that in adrenalectomized rats receiving DCA and salt alone the loss of N^{15} from serum protein occurred at the same rate as in similar animals receiving ACE and as in the intact controls.

It therefore appears that the integrity of the adrenal cortex is not essential for the production or the release of antibody.

In unpublished experiments, however, it was seen, in agreement with Dougherty, Chase and White,[10] that shortly after a single injection of ACE there occurred an elevation of serum antibody-N (of about 30%) in rabbits previously immunized with pneumococcus Type I. This nonspecific anamnestic rise lasted only about 12 hours and did not occur at all in animals whose previous antibody response had been relatively weak. Hence it appears that increased adrenal cortical activity may exert a transitory influence upon the distribution of preexisting antibodies. However, it is clear from the data presented above that adrenal cortical activity has no prolonged effect upon the production or release of antibodies and gamma globulin.

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[15] Selye, H., Stone, H., Nielson, K., and Leblond, C. P., *Canad. Med. Assn. J.*, 1945, **52**, 571.

[16] Knowlton, A. I., Stoerk, H. C., Seegal, B. C., and Loeb, E. N., *Endocrinology*, 1946, **38**, 315.

[17] Knowlton, A. I., Loeb, E. N., Stoerk, H. C., and Seegal, B. C., *J. Exp. Med.*, 1947, **85**, 187.

[18] Stoerk, H. C., and Zucker, T., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 297, and unpublished data.

[19] Heidelberger, M., Treffers, H. P., Schoenheimer, R., Ratner, S., and Rittenberg, D., *J. Biol. Chem.*, 1942, **144**, 555.

[20] Schoenheimer, R., Ratner, S., Rittenberg, D., and Heidelberger, M., *J. Biol. Chem.*, 1942, **144**, 545.

[21] Stoerk, H. C., John, H. M., and Eisen, H. N., in preparation.

Summary. Identical concentrations of serum antibodies and gamma globulin were found in adrenalectomized rats repeatedly injected during immunization with ACE and in similar animals not receiving ACE. It is concluded that adrenal cortical activity is not essential for the fabrication or release of antibodies and gamma globulin.

15943 P

Evaluation of Vagotomy in Chronic, Non-Specific Ulcerative Colitis.*

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Two previous papers by one of us[1,2] bear out the conclusion of Elsom and Ferguson[3] that surgical management of chronic, nonspecific, ulcerative colitis is safer and productive of better health than prolonged conservative treatment. The surgical management is beset with many pitfalls, however, and disastrous complications still occur.

In search of a simpler method of handling the problem, consideration has been given to division of the vagus nerves, with the thought in mind that decrease in general intestinal muscular activity might result and might ease the symptoms of the disease, as Dragstedt[4] has found to be the case in duodenal and gastric ulcers.

We were plunged into the problem by a patient who had had all her colon and ¾ of her small intestine resected for colitis and ascending ulcerative ileitis. Recurrent ileitis demanded some active therapy after all known conservative measures had failed. Vagotomy was suggested by Dr. C. J. Watson of the Department of Medicine, and resulted in marked improvement, the patient having thereafter the first solid stools she had had in 4 years. Unfortunately the improvement was temporary.

A plan was formulated to employ transthoracic vagotomy in a group of cases to determine the possible value of the procedure. Moore[5] had had a very poor result from vagotomy in one fairly severe case, and we therefore confined ourselves to minimally involved cases.

In the 4 cases in which vagotomy was the only surgical therapy, studies were performed before operation, in the 2 weeks afterward, and 6 weeks to 3 months after vagotomy. Observations include number and character of stools, general health, presence of blood, pus, mucus in the stools, proctoscopic and barium enema findings, transit time of material through the intestine by X-ray and charcoal, electrocardiograms, triple histamine (Lannin) gastric analyses, 12-hour night collection of gastric juice, and insulin gastric analyses with blood glucose checks.

As indicated in Table I, lasting good re-

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* Supported by a Research Grant from the Graduate School of the University of Minnesota.

[1] Dennis, Clarence, *Surgery*, 1945, **18**, 435.

[2] Dennis, Clarence, *Minnesota Med.*, 1945, **28**, 228.

[3] Elsom, K. A., and Ferguson, L. K., *Am. J. Med. Sci.*, 1941, **202**, 59.

[4] Dragstedt, L. R., *Surgery*, 1945, **17**, 742.

[5] Moore, F. D., *Bull. New Eng. Med. Center*, 1945, **7**, 52.

TABLE I.
Vagotomy in Chronic Ulcerative Colitis.

Case No.	Age	Duration, yrs	Complic.	Completeness of vagotomy	Results		
					Sympt.	Procto	X-ray
726638	20	7	6 ft. intest.	Incomplete	Temporarily excellent	——	——
647070	57	10	—	Complete	Excellent	Healed	Imp.
771995	14	2	Poliomyelitis	"	"	"	"
620076	57	13	——	"	Imp.	"	Neg. before
703015	44	5	D.U.	Incomplete	Equivocal	Neg. before	" "

sults have been obtained in those cases in which the insulin gastric analysis indicated that section of the vagus had been complete.

In the first case a severe tachycardia followed operation and persisted 2 weeks with electrocardiographic changes suggesting pericarditis. Electrocardiograms were therefore done before and after surgery to determine whether vagal section 4 to 5 cm above the diaphragm consistently cause electrocardiographic alterations. None were found except that one case has a sinus bradycardia (58 as against 70 preoperatively).

Stool examinations indicate that the pus, uniformly present in the stool preoperatively, disappeared postoperatively in 2 of those cases in which section was proved to be complete. In the third, pus was only occasionally present 3 months after operation, but some blood was consistently present.

Of particular interest is the transit time of material through the gut. No significant difference was observed except in the patient who had had 2 to 7 stools a day before surgery; in her case the transit time was 6 hours at that time. Four months after vagotomy it was 26 hours and she had dropped to one stool a day, formed.

Conclusion. Preliminary investigation indicates that vagotomy offers sufficient promise in chronic, nonspecific, ulcerative colitis to justify further investigation.

15944 P

Demonstration of a Positive Relationship Between Cardiac Output and Oxygen Consumption.*

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The purpose of this paper is to demonstrate a positive relationship between oxygen consumption and cardiac output. In order to accomplish this, a method of measuring cardiac output was needed in which the calculation of the cardiac output did not include the factor of oxygen consumption. For this reason the ballistocardiograph was selected, and Starr's[1] "height" formula was used on the tracings obtained on a high frequency table. The oxygen consumption was measured on the tracings obtained from a Benedict-Roth metabolism apparatus and all data was divided by the individual surface area.

Medical students, laboratory workers, and resident physicians were subjects of this study, and all were purposely studied under nonbasal conditions. Measurements of the

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* This work was carried out under a contract between the Office of Naval Research and the University of Rochester School of Medicine and Dentistry.

[1] Starr, I., and Schroeder, H. A., *J. Clin. Invest.*, 1940, **19**, 437.

TABLE I.

Data analyzed	No. cases	Coefficient of determination in %*	Correlation coefficient r	Significant	% change in output for each 10% increase in O_2 cons.
Cournand	34	51	.713	Yes	7.22
Brown and Pearson	42	30	.545	”	6.54
Stead and Warren	26	47.5	.689	”	5.92
Starr	48	16	.418	”	8.63

* The coefficient of determination denotes the amount of variability in cardiac output that can be explained by the one factor studied, namely oxygen consumption.

cardiac output were made just before and just after two 6-minute periods in which oxygen consumption was measured. The results were then averaged.

Forty-two records were then analyzed and a positive relationship obtained. The correlation coefficient was determined as .545, a significant relationship statistically. Furthermore, by calculating the regression equation the ratio of change was determined. It was found that for each 10% increase in oxygen consumption a 6.54% increase occurred in cardiac output.

To further substantiate this relationship, published data (using other methods of measuring cardiac output and oxygen consumption) were analyzed similarly. In these cases patients were under basal conditions Using data published by Cournand[2] in which cardiac output was determined on 34 cases employing right heart catheterization technic, and oxygen consumption by the analysis of expired air, a positive correlation coefficient of .713 was obtained. The regression equation was calculated and it was found that for a 10% increase in oxygen consumption a 7.22% increase occurred in cardiac output.

In 26 cases published by Stead[3] using the same procedures as Cournand for the determination of cardiac output and oxygen consumption a positive correlation coefficient of .689 was obtained. The regression equation revealed that for each 10% increase in oxygen consumption an increase of 5.92% occurred in cardiac output.

One other series of data was analyzed in which cardiac output was determined by the ethyl iodide technic and oxygen consumption from analysis of expired air. In 48 cases published by Starr[4] a positive correlation of .418 was obtained. Although, lower than the other methods, the value obtained is significant statistically. The calculation of the regression equation reveals that for a 10% increase in oxygen consumption an increase of 8.63% occurs in cardiac output.

A summary of the results appears in Table I.

The average value obtained by the 4 observers and 3 methods of measuring cardiac output shows that for each 10% increase in oxygen consumption a 7.07% increase occurs in cardiac output. Furthermore, the differences between the percentages tabulated are not significant statistically.

Conclusions. 1. Regardless of the methods used in measuring cardiac output and oxygen consumption a significant positive relationship was demonstrated for 3 different methods between oxygen consumption and cardiac output. 2. Maximum variation explicable in cardiac output by variation in oxygen consumption was approximately 50%. This coefficient of determination reveals that only one-half of the variance in cardiac output could be explained by changes in oxygen consumption. The amount of variation in cardiac output explicable by oxygen consumption might be improved if in addition to basal conditions, uniform environmental temperature and humidity were maintained during the study. 3. The calculation of the regression equation for each series of data demonstrates a striking constancy of change. Despite variance in methods for determining cardiac output and oxygen consumption, and varying basal or nonbasal conditions of the subjects, the ratios of change are the same. For each 10% increase in oxygen consumption an average increase of 7.07% in cardiac output results. 4. Relative changes in cardiac output are the same regardless of whether one uses the ballistocardiograph, right heart catheterization, or ethyl iodide method in the determination of cardiac output.

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[2] Cournand, A., Riley, R. L., Breed, E. S., Baldwin, E. deF., and Richards, D. W., Jr., *J. Clin. Invest.*, 1945, **24**, 106.

[3] Stead, E. A., and Warren, J. V., *J. Clin. Invest.*, 1945, **24**, 326.

[4] Starr, I., Collins, L. H., Jr., and Wood, F. C., *J. Clin. Invest.*, 1933, **12**, 13.

15945

The Culture of Tissue Cells in Clots Formed from Purified Bovine Fibrinogen and Thrombin.*

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The plasma clot has long been accepted as the most satisfactory support for the growth of tissue cells *in vitro*. Presumably the strands of fibrin form a meshwork along which the cells migrate and by which they maintain a distribution and spacing which permits the free intercellular diffusion of nutrients and metabolites. Regardless of the precise relationship between cells and clot, their growth in the plasma coagulum is better than on any other supporting surface.[1]

As the source of coagulum, chicken plasma has been most favored. The clot formed is firm and transparent and is more resistant to the lytic effect of tissue explants, particularly those of heterologous origin, than are clots of other whole plasmas.[2-4] There are, however, several undesirable features connected with its use. There are obvious problems inherent in the collection and storage of chicken plasma. There are considerable variations in clotting properties and in the influence of the plasma on culture growth which apparently are related to the age and health of the donor.[5] Whole plasma is, moreover, of complex and, to a great extent, unknown composition. Finally, it can be the medium of transmission of infectious agents, especially viruses.[6-9] What is required, then, is the source of a clot or its equivalent more conveniently obtained than chicken plasma, free of complicating infectious agents, and acceptable to the cells as a supporting structure.

The methods for the fractionation of plasma proteins developed in the Department of Physical Chemistry of the Harvard Medical School have made available many of the proteins of both human and bovine blood plasma in states of greater purity and in larger amounts than could have been obtained readily by previous methods.[10–13] To a great extent, clots formed from 2 of these, fibrinogen and thrombin, meet the requirements of the tissue culture technic. Fibrinogen is not so complex as whole plasma and its degradation products are known approximately.[14] It is obtainable in large quantities in a stable form and so can be considered as constant for any long series of experiments. It is unlikely to carry any viruses. By the use of these purified clotting substances and by controlling simple conditions under which clotting takes place, the investigator may form clots of varied mechanical and optical properties suited to the problem at hand. These, and other considerations related to the technical use of these purified proteins are the substance of this report.

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* The initial results of these studies were described at the Hershey Conference on Tissue Culture, sponsored by the Committee on Growth of the National Research Council and held at Hershey, Pa., November 10-13, 1946.

† Junior Fellow, The Society of Fellows, Harvard University, Cambridge, Mass.

[1] Fischer, A., *Biology of Tissue Cells*, Copenhagen, Gyldendalske Boghandel Nordisk Forlag, 1946.

[2] Parker, R. C., *Methods of Tissue Culture*, New York, Paul B. Hoeber, Inc., 1938.

[3] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1928, **48**, 105.

[4] Santesson, L., *Acta Path. et Microbiol. Scand.*, Suppl. 24, 1935.

[5] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, **34**, 599.

[6] Furth, J., *J. Exp. Med.*, 1932, **55**, 465.

[7] Doyle, T. M., *J. Comp. Path. and Therap.*, 1927, **40**, 144.

[8] Todd, C., *Brit. J. Exp. Path.*, 1928, **9**, 19.

[9] Burmeister, B. R., Prickett, C. O., and Belding, T. C., *Cancer Res.*, 1946, **6**, 189.

Materials and Methods. a. *Preparation of Solutions.* The fibrinogen used in these studies was that contained in Fraction I of bovine blood plasma,[‡] prepared according to the methods of Cohn and his associates.[13] A 0.5% solution of the fibrinogen in Tyrode's solution[§] has been found a convenient concentration to use. Sterilization, if necessary, is easily accomplished by pressure filtration through a Seitz asbestos filter. The filtrate can be stored at 0°C for several weeks without apparent loss of clotting properties.

The thrombin preparation used to bring about polymerization of the fibrinogen was that contained in Fractions II and III of bovine plasma.[‖] The sterile dry powder was dissolved in Tyrode's solution to give a final concentration of 10 to 20 units/cc. This concentration is convenient because one drop will contain 0.5 to 1 unit of thrombin which is sufficient to clot 1 cc of a fibrinogen-nutrient mixture in a culture flask and to allow enough time for orientation of the tissue explants. It has been found that at 0°C thrombin in solution loses its activity quite rapidly and is not of value after 5 or 6 weeks. However, at the lower temperature of "dry ice" storage (−50°C to −70°C) the property of eliciting clot formation appears to be retained unaltered over several months. This seems to be a convenient as well as an economical method for making thrombin in solution readily available.

b. *Preparation of Clots.* The following procedure is presented as one that has been found satisfactory for the preparation of cultures with clots from purified fibrinogen. It has been used only in flask cultures and only with flasks adapted for the roller-tube rack.[17] Regular Carrel flasks could be used in the

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[10] Cohn, E. J., McMeekin, T. L., Oncley, J. L., Newell, J. M., and Hughes, W. L., *J. Am. Chem. Soc.*, 1940, **62**, 3386.

[11] McMeekin, T. L., *J. Am. Chem. Soc.*, 1940, **62**, 3393.

[12] Cohn, E. J., Luetscher, J. A., Oncley, J. L., Armstrong, S. H., Jr., and Davis, B. D., *J. Am. Chem. Soc.*, 1940, **62**, 3396.

[13] Cohn, E. J., Strong, L. E., Hughes, W. L., Jr., Mulford, D. J., Ashworth, J. N., Melin, M., and Taylor, H. L., *J. Am. Chem. Soc.*, 1946, **68**, 459.

[14] Brand, E., Kassell, B., and Saidel, L. J., *J. Clin. Invest.*, 1944, **23**, 437.

[‡] We are indebted to Armour and Company of Chicago, Ill., Chemical Research and Development Department for a generous supply of Fraction I (Lot C-185) of bovine plasma. Of the total protein of this lot, approximately 70% has been shown to be fibrinogen. Preparations of greater purity can be obtained, but for preliminary experiments they did not seem essential. This preparation is distributed in the form of a dry powder. In addition to the fibrinogen there are also present small quantities of the other plasma proteins and a portion of sodium citrate (about 40% of the dry weight).

[§] Earle's modification of Tyrode's solution.[15]

[15] Earle, W. R., *J. Nat. Cancer Inst.*, 1943, **4**, 165.

[‖] A Parke-Davis preparation of thrombin designed for topical application was used. This can be obtained commercially as a sterile preparation, distributed as a dry powder in small glass ampoules containing approximately 5000 units. The unit is defined as the amount required to clot 1 cc of a standard fibrinogen solution in 15 seconds,[16] or more precisely, the amount which clots a 1% solution of fibrinogen in a test tube 1 cm in diameter at 25°C, pH 6.3, in approximately 45 seconds.[18]

[16] Seegers, W. H., Smith, H. P., Warner, E. D., and Brinkhous, K. M., *J. Biol. Chem.*, 1938, **123**, 751.

[17] Porter, K. R., Claude, A., and Fullam, E. F., *J. Exp. Med.*, 1945, **81**, 233.

same way, but for roller tube and slide cultures other procedures would, of course, be necessary.

The tissue explants are first placed in the neck of the tube, close to the flask portion. All the excess Tyrode's solution is withdrawn from around them. The fibrinogen solution (0.5% or 0.25%) and nutrient¶ are pipetted to give a total volume of one cubic centimeter. One drop of thrombin solution (containing 0.5 to 1 unit) is next added and all the ingredients are mixed by gentle shaking. The flask is quickly placed in a horizontal position and the explants are oriented. At room temperature clotting will take place in a minute or less. The resulting coagulum is rigid and fairly transparent. In a few hours at 38°C it will undergo syneresis and exude about one-half of its volume.

Recent detailed studies on the clotting mechanism have been reported by Ferry and Morrison.[18] They showed that such characteristics of experimental clots as degree of opacity, rigidity and syneresis could be varied within wide limits by the control of such conditions as hydrogen ion concentration, temperature, and concentrations of the reacting proteins. These investigators used fibrinogen and thrombin preparations of human origin. We have repeated certain of their experiments of special interest to those concerned with tissue cultures, and have used the above described bovine proteins. The results were essentially similar to those obtained with human fibrinogen and thrombin and form the basis of the following recommendations.

If one desires a transparent clot like that obtained from chicken plasma, it is important to have the solutions at room temperature (20°C). Ice-cooled nutrient and fibrinogen solutions give a rather opaque clot with inferior optical properties. If the pH of the solution is below 7.5 during clot formation, the opacity is appreciable and can be shown to increase as the hydrogen ion concentration increases. A clot of satisfactory transparency is readily obtained by having the fibrinogen-nutrient mixture at pH 7.6 to 7.8. After clotting has been completed, the pH of the nutrient may be changed without loss of transparency of the formed clot.

Thrombin concentration, varied over a wide range, does not greatly influence the transparency of the clot. In these studies it has been reduced to as low a concentration as 0.1 unit per cc of fibrinogen-nutrient mixture and yet a clot was obtained.** This was slightly opaque, but not enough to make it unsuitable, and it supported good cell growth. Clotting was, of course, greatly delayed, 10 minutes being required, but for some types of culturing a large portion of this time might be needed. With such a wide range of concentrations effective in providing transparent clots, the concentration recommended is, therefore, that which gives the operator sufficient time to arrange the tissue explants.

c. *Cell Growth in Clots from Purified Fibrinogen.* The ability of the clot from purified fibrinogen to support the growth and migration of cells is, of course, the real measure of its usefulness. In these studies this has been examined with rat fibroblasts, rat myoblasts, Jensen rat sarcoma cells†† and chick fibroblasts. In general, the rate of growth observed, whenever the clot has not undergone lysis, has been similar to that obtained in chick plasma (Fig. 1). Minor differences in cell structure and colony archi-

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¶ This is composed of Tyrode's solution (5 parts), placental cord serum (3 parts), and embryo extract (2 parts). Using 2 parts of nutrient to one of fibrinogen solution further dilutes the latter so that the final concentrations of fibrinogen are approximately 0.08 to 0.16%.

[18] Ferry, J. D., and Morrison, P. R., *J. Am. Chem. Soc.*, 1947, **69**, 388.

** Calculations based on the dry weight of the original material show that by way of the solution about 0.025 mg is ordinarily added to a culture and that 0.005 mg is more than enough. When it is considered that only a small fraction of this is thrombin some impression is gained of the extreme dilution at which thrombin is active.

†† This tumor was kindly provided by Dr. R. L. Ladd, Northwestern University.

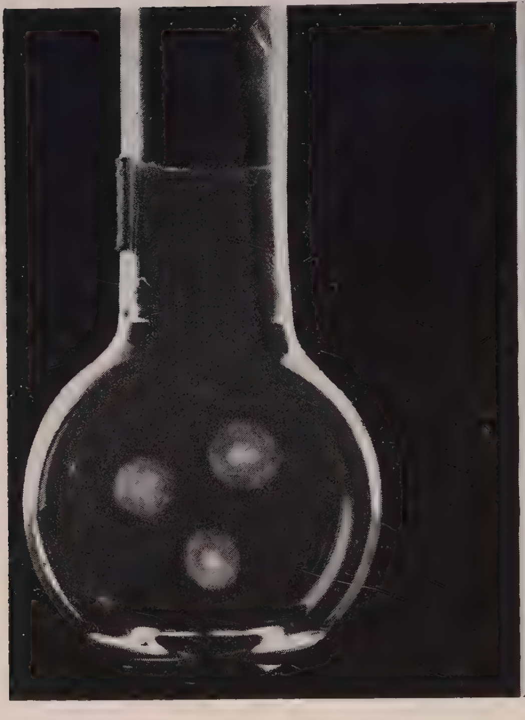

FIG. 1a.

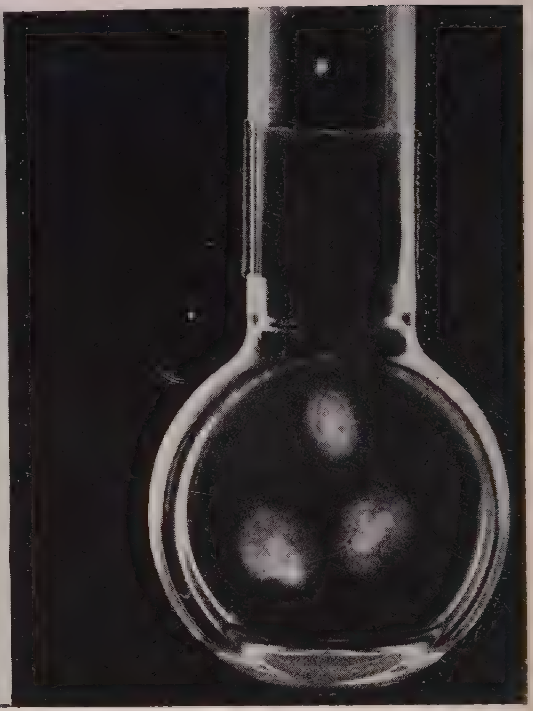

FIG. 1b.

FIG. 1a. Colonies of cells from skeletal muscle of chick embryo growing in clot from chick plasma.

FIG. 1b. Similar colonies of the same age growing in clot from bovine fibrinogen.

tecture have been noted, but these are not important to this report. It seems fairly certain that as the material is better understood and its possibilities and behaviors have been further examined, it will be accepted as a useful substitute for the clot from whole plasma.

Of the several conditions which doubtless favor or discourage the growth and migration of cells in these pure fibrin clots, the concentration of fibrinogen is of first importance. In our experience, if the final concentration is much greater than 0.16%, the clot is so dense that the cells have difficulty in making their way through it. Under such conditions the greatest migration is on the surface of the clot. Some cells push through but their pseudopodia show very fine arborizations very much as though they are trying to extend through extremely small spaces. Thus, the concentrations of fibrinogen supporting the most typical cell colonies have been 0.16% and 0.08%. Lower concentrations have not been tested.

Experiment 1. Cultures of rat fibroblasts from explants of one-day-old rat skeletal muscle were set up in clots containing 2 parts of nutrient (5:3:2) and 1 part of fibrinogen solutions. The latter was used in 3 different concentrations, 1.0%, 0.5% and 0.25% in Tyrode's, to give final concentrations in the nutrient-fibrinogen mixtures of 0.33%, 0.16% and 0.08% of clotting fibrin. Four flask cultures, each with 3 explants, were prepared with each concentration of fibrinogen. To bring about clotting, one drop of Tyrode's solution of thrombin containing 20 units per cc was added to each culture. Control cultures were simultaneously prepared with 2 parts of nutrient and 1 part whole chick plasma. The cultures were each fed twice weekly for 3 weeks with 1 cc of the 5:3:2 nutrient mixture and during this time comparisons were made of cell growth and migration and colony structure.

In the 0.33% fibrin clot, migration of the

TABLE I.
Measurement of Growth by Increase in Width of Colony Margin.

Age of culture	Avg for controls mm	Avg for cultures in 0.16% fibrin, mm	Avg for cultures in 0.08% fibrin, mm
4	1.5	1.3	1.2
6	2.0	1.8	2.0
8	3.1	3.1	3.2
10	3.5	4.0	4.2
12	4.0	5.0	5.6
14	4.0	5.2	6.2

cells was always restrained relative to behavior in control cultures. It appeared that the cells were not able to find a way through the clot. Their pseudopodia developed fine arborizations, as though probing the small interstices. After one week, long cords of cells having the appearance of hyphae of mold mycelia could be seen, but the best migration was only on the upper and lower surfaces of the coagulum.

Growth and migration of cells in the 0.16% fibrin clot approached more nearly their extent in the control cultures. Hyphae-like filaments of cells appeared but were more numerous and more branched. After 3 weeks the growth in these cultures was almost as extensive as in the controls.

The cultures having clots with a concentration of 0.08% fibrin developed at essentially the same rate as the controls and the cells showed no striking abnormalities. After 2 weeks, the clots of these cultures began to undergo lysis but the process never became extensive or interfered with the continued growth of the cultures.

Another experiment, similar to the above, was carried out with cells of the Jensen rat sarcoma. In this the fibrin content of the clot was varied from 0.33% to 1.0%. Migration of cells was possible only through the clot made from the lower concentration (0.33%) and even in this the cells extended from the explant as columns of more or less cuboidal units.

It has been noted that, as might be expected, growth starts up more promptly after making the culture if the nutrient is incorporated in the clot. In some cultures, for example, explants were set up in clots developed solely from fibrinogen in Tyrode's solution. The nutrient mixture was then added as a liquid phase above the clot. The initial growth here was poor‡‡ but after a week or 10 days it had overtaken the other cultures started in the usual manner.

If the concentration of fibrin is suitable and other nutritional conditions are the same, the migration and growth of cells in clots of fibrinogen origin seem to be essentially equal to that in chick plasma (Fig. 1). An experiment made with fibroblasts from skeletal muscle of an 11-day-old chick embryo illustrates this.

Experiment 2. Cultures of chick embryo skeletal muscle were set up as follows:

4 flasks—3 explants per flask with 1 part chick plasma, 2 parts nutrient (5:3:2).
4 flasks—3 explants per flask, 1 part fibrinogen 0.5% and 2 parts nutrient (5:3:2).
4 flasks—3 explants per flask, 1 part fibrinogen 0.25% and 2 parts nutrient (5:3:2).

Twenty-four hours after preparation each culture was given 1 cc of nutrient and this was changed every 3 or 4 days thereafter during the course of the experiment. The cultures were examined every 2 days and measurements were made of the width of the margin of cell migration and growth (Table I).

This type of measurement, while only an approximation, was considered adequate for the purpose of this experiment. Its significance was further limited by the observation that the population of cells in the growth margin of the control colonies seemed more dense than in the colonies in the fibrinogen clots. There was a limited lysis of plasma clot in one case, but no lysis of any of the fibrinogen clots.

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‡‡ This could be related to absence of nutrients or to the presence of considerable sodium citrate.

Besides demonstrating the suitable nature of fibrinogen clots for culturing cells of the kind studied, these data probably have little significance. The greater width of the growth margin in the fibrinogen clots may be related to a less dense population of cells and the thinner nature of the fibrinogen clot which seems to flatten more than the plasma clot during syneresis. Possibly also of influence in this respect is the absence of, or the delayed appearance of any change in the character of the purified fibrinogen clot. As is well known, it is not uncommon for the clot from whole plasma to become very opaque around the margin after 2 or 3 weeks in a flask culture. It has the appearance of denatured fibrin. This same transformation, which may restrict cell migration and colony expansion has not, in our experience, appeared at all in the clots from bovine fibrinogen.

In the transferring of tissue cultures the properties of the fibrinogen coagulum are of some importance and so have been examined. Up to the time of this report, flask cultures of rat fibroblasts and also of chick fibroblasts in fibrinogen clots have been transferred for 3 generations with as much ease and success as their equivalents in chick plasma coagula. They are not more friable on transfer; if anything, less so.

From these limited observations it appears that the purified fibrinogen clot does not resist lysis as completely as the chick plasma clot. In this respect, however, it seems similar to coagula derived from many mammalian sources. Lysis was observed to be most pronounced in the presence of rat myoblasts (or explants of young rat hearts) and less so with rat sarcoma (Jensen) cells. Growth of rat fibroblasts from explants of skeletal muscle was supported for 2 weeks without any liquefaction and it appeared then only in clots derived from the more dilute preparations of fibrinogen. Finally, the combination of explants of the skeletal muscle of chick embryos and bovine fibrinogen gave excellent cultures which showed no lysis at all. These observations, together with recorded notes on clot lysis in tissue cultures,[4] suggest that the process might be controlled to some extent by proper selection of the source of the purified fibrinogen. For example, fibrinogen prepared from chick plasma might generally prove more resistant to the lytic activity of mammalian cells than does fibrinogen from bovine sources. There may, in addition, be other methods of control, either through the selection of appropriate sera for the nutrient or by suitably treating the fibrin during or after its polymerization. Studies on this problem are being continued.

It may be shown subsequently that the size of the fibrin strands in a clot, as well as the size of the interstices, are influential in determining cell migration and even multiplication. Possibly the finer strands and smaller spaces inhibit these cell functions. If this is found to be true, it may be necessary to accept a clot of partial opacity in order to obtain larger fiber size; for it has been shown that the degree of opacity is determined by fiber size.[18,§§]

Summary. Procedures are described for the successful culture of tissue cells in clots formed from purified bovine fibrinogen and thrombin. The influence on clot characteristics of pH and temperature, effective during clot formation, and the influence of fibrinogen concentration on the subsequent cell migration in the clot are indicated. Cultures grown in these clots can be transferred with the same ease as those grown in clots derived from chick plasma. The problem of fibrinolysis is briefly mentioned.

[§§] An electron microscope study of the structure of the fibrin clot is being made by the authors. There is a marked increase in fiber size in opaque clots formed at pH 6.5 as compared with fiber size in transparent clots formed at pH 8.5. Preliminary measurements show the fibers in the clot formed at the more acid pH to range roughly from 50 to 350 μ, at the more alkaline pH they vary from 15 to 90 μ. Also of interest is the presence of striations across the fibrin strands which show a surprisingly constant periodicity of 240 Å at pH 6.5.

15946

Niacin Deficiency in Rabbits and Response to Tryptophane and to Niacin.

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Tryptophane has been shown to be effective in curing niacin deficiencies in the rat,[1] the chick,[2] the mouse,[3] and in the dog.[4]

Wooley and Sebrell[5] showed that growing rabbits fed a purified diet containing 20% casein, required niacin for survival and growth. The only symptom attributed to niacin deficiency was anorexia.

The study reported below concerns the response of niacin-deficient rabbits to tryptophane as compared to niacin.

Experimental. Twenty-four male, New Zealand rabbits, about 3 months of age, in litters of from 2 to 8, and weighing between 1600 and 2000 g each, were placed in metal cages with wire mesh floors. The animals were divided into 4 groups of 6 each, separating the littermates. All of these rabbits were fed purified diet No. 673, previously described.[5] Assays of the diet showed less than 0.05 μg of niacin per gram.

After 8 weeks on the basal diet, the experimental animals were treated for 4 weeks as follows:

Group 1. Continued on basal diet.

Group 2. 400 mg *dl*-tryptophane per rabbit per day mixed into the food.

Group 3. 10 mg niacin per kg body weight per rabbit per day mixed into the food.

Group 4. Received both tryptophane and niacin as in Groups 2 and 3.

Two rabbits, one in Group 1 and one in Group 3, died before the study was completed.

The weight of each rabbit and the amount of ration consumed was recorded weekly. Hemoglobin and hematocrit determinations, and total leukocyte and differential counts were made at the end of the 2 periods of observation.

All of the animals were sacrificed at the end of the experiment. The liver weights were recorded. Portions of the liver and hamstring muscles from each rabbit were analyzed for total nitrogen,* niacin and tryptophane. The niacin was determined by the author's modification of the method of Snell and Wright.[6] The casein hydrolysate in the medium was increased to 10%, sodium acetate to 2%, and dextrose to 2.5%. The niacin standard curve contained from 0.2 to 2 μg per tube. The tryptophane was determined by the method described by Wooley and Sebrell.[7]

Results. Weight gains and food consumption are recorded in Table I. Rabbits fed the basal diet ate an average of 250 g per week and lost an average of 90 g during the first 8-week period.

During the next period of 4 weeks, the rabbits that were continued on the basal ration showed a decline in appetite and a more rapid loss of weight. The animals that were fed niacin and those that were given tryptophane gained weight and ate increased amounts of ration at a similar rate. Those that received both showed greater weight gains and increases in food consumption than did the animals that received either of these supplements alone.

Blood changes are summarized in Table II. All of the rabbits were anemic at the end of 8 weeks on the basal ration. Most of them showed low leukocyte and granulocyte counts. At the end of the next 4 weeks, the rabbits

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[1] Krehl, W. A., Teply, L. J., Sarma, P. S., and Elvehjem, C. A., *Science*, 1945, **101**, 489.

[2] Briggs, S. M., *J. Biol. Chem.*, 1945, **161**, 749.

[3] Woolley, D. W., *J. Biol. Chem.*, 1946, **162**, 179.

[4] Hundley, J. M., personal communication.

[5] Wooley, J. G., and Sebrell, W. H., *J. Nutrition*, 1945, **29**, 191.

[6] Snell, E. E., and Wright, L. D., *J. Biol. Chem.*, 1941, **139**, 675.

[7] Wooley, J. G., and Sebrell, W. H., *J. Biol. Chem.*, 1945, **157**, 141.

* The author is indebted to Charles A. Kinser, Chemist, National Institute of Health, for these determinations.

TABLE I.
Average Weight and Food Consumption.

No. of rabbits		Original wt	Deficiency period: After 8 wk, wt	Deficiency period: Avg weekly food consumption	Treatment period: After 4 wk, wt	Treatment period: Avg weekly food consumption
			Group 1. No treatment given.			
5	Avg	1850	1780	248	1630	184
			Group 2. Treated with tryptophane.			
6	,,	1760	1660	254	1990	316
			Group 3. Treated with niacin.			
5	,,	1730	1600	260	1910	326
			Group 4. Treated with tryptophane and niacin.			
6	,,	1790	1730	245	2190	398

TABLE II.
Blood Findings.

No. of rabbits		After 8 wk deficiency period: Hemoglobin	Hematocrit	Leukocytes	Granulocytes	After 4 wk treatment period: Hemoglobin	Hematocrit	Leukocytes	Granulocytes
				Group 1. No treatment given.					
5	Avg	9.5	27.4	8120	1680	8.9	25.8	6410	2230
				Group 2. Treated with tryptophane.					
6	,,	9.3	27.3	6270	1725	11.9	37.4	9160	4090
				Group 3. Treated with niacin.					
5	,,	9.4	28.4	5330	1790	11.7	34.1	8200	3270
				Group 4. Treated with tryptophane and niacin.					
6	,,	10.1	29.4	7810	1540	13.2	38.7	9170	2910

that were continued on the basal diet were found to have still lower hemoglobin, hematocrit, and total leukocyte values. The granulocytes showed some increase, but did not reach the levels of the treated animals. The hemoglobin and hematocrit values, and the total leukocyte and granulocyte counts increased significantly in all of the treated groups.

The livers of the rabbits that were continued on the basal diet weighed less than did the livers of the supplemented animals.

The niacin content of the livers of rabbits fed the basal ration averaged 116 μg per g. This amount was from 43 to 70 μg less than the average amount found in the supplemented animals. The livers of the animals that received both tryptophane and niacin averaged the highest concentration of niacin.

The niacin content of the muscles of the rabbits in the various groups showed a different pattern from that of the livers. The muscles of the unsupplemented animals, and those of the rabbits that were fed tryptophane, showed similar assay values, *viz.* 88 and 93 μg per g. The muscles of the rabbits that received niacin and those that received both niacin and tryptophane in their diets averaged higher values but they were similar to each other, *viz.* 130 and 128 μg per g, respectively.

The tryptophane and the nitrogen values of the livers and of the muscles of the rabbits in the experimental groups showed very little differences.

Summary. In the study reported above, it is shown that the exclusion of niacin from the ration of rabbits for 8 weeks was accompanied by a reduction of hemoglobin and the cellular components of the blood. After 12 weeks on this ration, there was a reduction of niacin concentration in the livers and the hamstring muscles.

After the animals had been on the niacin-deficient ration for 8 weeks, attempts to cure the deficiency by feeding tryptophane or, and niacin for 4 weeks were made. The addition of 400 mg *dl*-tryptophane per rabbit or 10 mg niacin per kg body weight to the daily ration of the animals was followed by increases in appetites. The responses in weight

TABLE III.
Assay of Livers.

No. of rabbits		Wt of livers	Niacin, µg per g	% protein	Tryptophane % in protein
Group 1. No treatment given.					
5	Avg	54	116	14.2	1.60
Group 2. Treated with tryptophane.					
6	”	82	177	15.0	1.56
Group 3. Treated with niacin.					
5	”	78	159	13.4	1.57
Group 4. Treated with tryptophane and niacin.					
6	”	102	187	14.6	1.81

TABLE IV.
Assay of Muscles.

No. of rabbits		Niacin, µg per g	% protein	Tryptophane % in protein
Group 1. No treatment given.				
5	Avg	88	18.5	1.43
Group 2. Treated with tryptophane.				
6	”	93	19.2	1.43
Group 3. Treated with niacin.				
5	”	130	19.1	1.44
Group 4. Treated with tryptophane and niacin.				
6	”	128	20.7	1.47

gain, hemoglobin, red and white blood cells were about equal when either of the substances were fed. The addition of both compounds to the ration resulted in greater responses. The niacin content of the livers of the rabbits that received either tryptophane or niacin supplements was approximately normal. The niacin in the muscles of the rabbits that were supplemented with niacin was found to be high, while the tryptophane animals had a niacin level similar to the concentration found in the muscles of the deficient rabbits.

15947

Effect of Hypophyseal Growth Hormone Upon Rats Fed Low Protein Diets.*

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In a current study[1] the degree of nitrogen retention produced by the hypophyseal growth hormone has been shown to depend on the dietary protein content. With diets containing 12, 18, 24 and 48% casein growth hormone produces prompt nitrogen retention and proportionate gain of weight. In this study adult female rats of the Long-Evans strain, aged 7 to 10 months, were each fed 12 g daily of a synthetic diet complete in all known dietary requirements with a protein content restricted to 6% alcohol-washed casein. This diet contains 0.1% choline

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* Aided by grants from the Research Board of the University of California and the Rockefeller Foundation of New York City.

[1] Gordan, G. S., Bennett, L. L., Li, C. H., and Evans, H. M., to be published.

TABLE I.
Effects of Growth Hormone on Body Weight and Urinary Nitrogen Excretion of Rats on 6% Casein Diet.

	10 days preinj. period	5 days inj. period	Increment	10 days postinj. period
Controls				
Wt	249 g	248 g	—1 g	251 g
N	76±1.4 mg	77±1.6 mg		76±1.9 mg
Growth hormone injected				
Wt	251 g	252 g	+1 g	256 g
N	79±2.4 mg	62±3.2 mg	15 mg p* = <0.01	78±2.1 mg

* Fisher's[3] probability value.

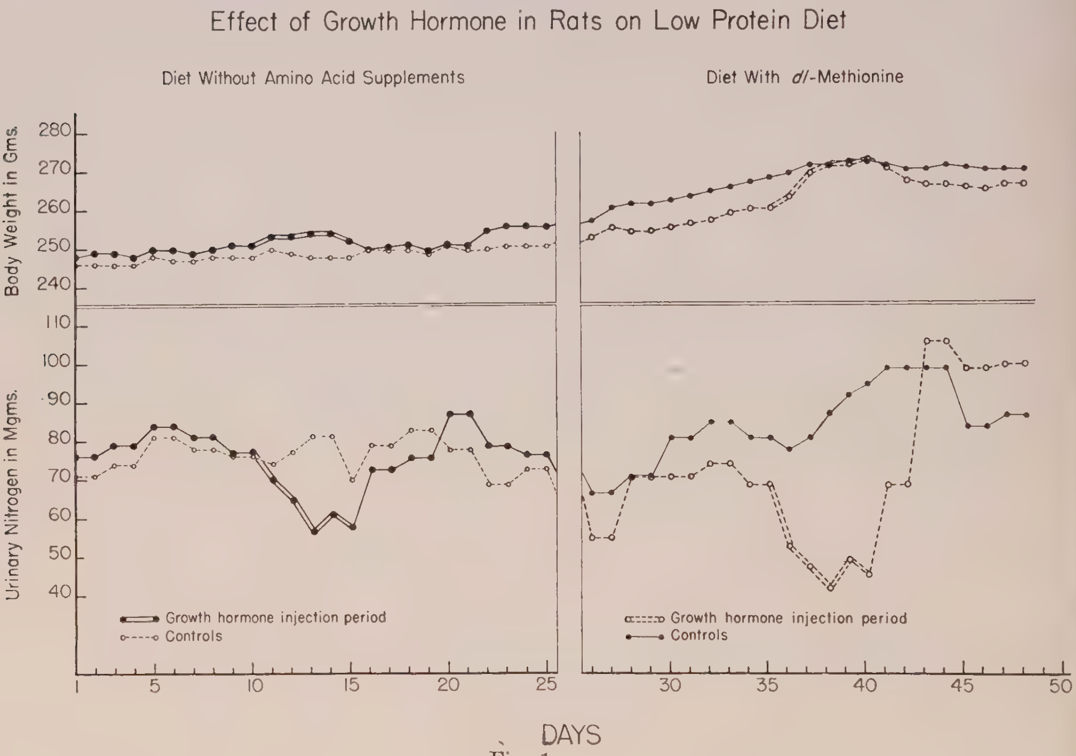

Fig. 1.

chloride. Animals have been fed this diet for 12 weeks without losing weight or developing hyperazoturia. The only sign observed which was considered evidence of a deficiency condition was loss of fur, especially that of white animals, most marked on the lower belly and flanks.

After a 7-week adaptation period on this diet rats were placed in individual screen-bottomed cages. Urines were collected through acid-moistened ribbed glass funnels in which feces and hair were separated upon perforated porcelain discs. The funnels were washed down and urines collected at 48-hour intervals during control periods and at 24-hour intervals during experimental periods. Urines were analyzed for nitrogen content by the micro-Kjeldahl method. Animals were weighed at the same time daily immediately before feeding.

After a 10-day control period 5 experimental animals were injected intraperitoneal-

TABLE II.
Effect of Growth Hormone on Body Weight and Urinary Nitrogen Excretion of Rats on 6% Casein Diet Plus *dl*-Methionine.

	10 days preinj. period	5 days inj. period	Increment	8 days postinj. period
Controls				
Wt	269 g	273 g	+4 g	271 g
N	77±2.3 mg	87±2.4 mg		92±2.0 mg
Growth hormone injected				
Wt	261 g	274 g	+13 g	267 g
N	60±2.3 mg	48±2.6 mg	39 mg $p = <0.01$	92±3.9 mg

ly with 0.5 mg of growth hormone[2] twice daily for 5 days while 5 control animals were similarly injected with an equal amount of serum albumen. Following injection the animals were observed for a second 10-day control period. The results of this experiment are presented in Table I and Fig. 1.

It will be noted that the growth hormone-injected rats retained nitrogen but showed no significant weight gain. This experiment has been repeated in 2 further series with similar results.

Following the second control period all animals received a dietary supplement of *dl*-methionine 66 mg per animal per day. By reference to Fig. 1, it will be noted that there was an immediate but transient retention of nitrogen and the animals gained weight. During this period the loss of hair also ceased and fur began to grow again in thinned out areas.

Following a 10-day control period the animals which had formerly acted as controls were injected intraperitoneally with 0.5 mg of growth hormone twice daily while the former experimental group served as controls.

By reference to Table II and Fig. 1 it will be noted that on the methionine-supplemented diet the growth hormone-treated rats retained nitrogen and gained weight. This experiment has been repeated in a further series fed the methionine-supplemented diet with similar results (nitrogen retention 32 mg; weight gain 7 g). Another series in which the diet was supplemented with *dl*-methionine 66 mg and *l*-tryptophane 22 mg per rat per day showed similar nitrogen retention (39 mg) and weight gain (7 g). In one series *l*-tryptophane 22 mg per day alone was used as the dietary supplement. When this group of rats was injected with growth hormone, there was an average nitrogen retention of 33 mg per day but no change of weight.

It thus appears that the addition of *dl*-methionine specifically supports the 6% casein diet to render it capable of supporting the rapid growth induced by growth hormone. The action of methionine is probably related to the amino acid requirement for building new tissue rather than the repair of hepatic damage produced by the low protein diet, for no histological difference is noted between the livers of rats on the unsupplemented diet and those of rats on the methionine-supplemented diet.

Conclusions. (1) The injection of growth hormone into rats fed a 6% casein diet results in nitrogen retention without significant gain in weight. (2) The addition of *dl*-methionine renders the 6% casein diet capable of supporting the rapid growth induced by the growth hormone.

We wish to acknowledge the receipt of generous amounts of vitamins from Hoffmann-La Roche Company, Nutley, N. J., Merck and Company, Inc., Rahway, N.J., and Lederle Laboratories, Inc., Pearl River, N.Y.

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[2] Li, C. H., and Evans, H. M., *Science*, 1944, **99**, 183.

[3] Fisher, R. A., *Statistical Methods for Research Workers*, 10th edition, Edinburgh, London, Oliver and Boyd, 1946.

15948 P

The Isolation of Morphologically Intact Mitochondria from Rat Liver.*

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In previous communications it was reported that the respiratory enzyme systems, cytochrome oxidase and succinoxidase, were associated, probably exclusively, with the "large granule" fraction isolated by differential centrifugation either from water homogenates[1] or from saline extracts[2] of rat liver. The identification of the components making up the large granule fraction presented an important and difficult problem. Microscopical examination of the fraction prepared in isotonic saline revealed the presence of spherical bodies ranging between 0.5 to 2 μ in diameter. The corresponding preparation obtained from a water homogenate of liver contained much larger and paler spheres. It was suggested, mainly on the basis of a comparison of the size of the isolated granules with that of known cellular inclusions, that the large granule fraction consisted mostly of mitochondria.[2,3] It was realized, however, that this evidence was not conclusive proof for the hypothesis that large granules were mitochondria, since the isolated large granules did not possess the generally accepted properties of mitochondria, namely, a rod-like shape and the ability to stain vitally with Janus green.

Subsequent investigations showed that isotonic saline solutions were undesirable media for reasons other than the above cytological considerations, in that sodium chloride, as well as certain other electrolytes, caused agglutination of large granules. This agglutination was reflected in the finding that irregular and usually great amounts of succinoxidase activity were sedimented during the removal, by low-speed centrifugation, of nuclei from saline homogenates. When water was used as the medium, no agglutination was visible, and the loss of succinoxidase in the nuclear fraction was much less.

The question whether the respiratory enzymes are actually associated with mitochondria was thus resolved into the search for a medium that would preserve the morphological and cytological characteristics of mitochondria and permit their isolation in good yields. It was found initially that agglutination did not occur in homogenates prepared in isotonic sucrose (0.25 M). Further studies of the effect of sucrose at different concentrations led to the surprising observation that as the concentration in a series of homogenates was increased, an increasing proportion of the large granules were distinctly rod-like in shape, until at a concentration of 0.80-1.0 M sucrose they could not be distinguished morphologically from the mitochondria of the living cell.

The isolation of morphologically intact mitochondria was effected as follows. Rat liver was homogenized in an all-glass apparatus[4] in 0.88 M sucrose (final liver concentration was 1.0 g in 10 ml homogenate). Free nuclei and residual intact cells were first removed completely by centrifuging the homogenate 3 times at 600 × gravity for 10 minutes. Centrifugation of the supernatant for 20 minutes at 24,000 × gravity resulted in sedimentation of all the free mitochondria, together with a small number of "microsomes."[3] The latter remained in the supernatant when the mitochondria were resuspended in 0.88 M sucrose and resedimented at 24,000 × gravity. Resuspension of this final sediment in 0.88 M sucrose yielded a distinctly yellowish preparation that showed pronounced birefringence of flow and was made up of mitochondria that had retained their original rod-like shape. The washed mitochondria were readily stained with Janus green at a dye concentration of 1/20,000, perceptibly stained at a dye concentration of 1/40,000, and remained morphologically stable in 0.88 M sucrose over a period of several days when kept at 4°C. No extraneous elements could be seen either in preparations stained with Janus green or in preparations fixed with osmium tetroxide and examined at high magnification in the electron microscope. In several experiments, the suspensions of washed mitochondria isolated by this procedure were found to contain 70 to 80% of the succinoxidase activity of the original liver homogenate, the remainder of the enzyme activity being present in the mixture of nuclei and unbroken cells sedimented by the preliminary low-speed centrifugations.

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* Aided in part by a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

† Fellow of the Jane Coffin Childs Memorial Fund for Medical Research.

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[2] Hogeboom, G. H., Claude, A., and Hotchkiss, R. D., *J. Biol. Chem.*, 1946, **165**, 615.

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Of some interest is the fact that results obtained with rat kidney homogenates in sucrose solutions paralleled those described above for liver. Furthermore, the morphological alteration of mitochondria within unbroken liver or kidney cells present in homogenates, a phenomenon that occurred very rapidly in isotonic saline or 0.25 M sucrose, was progressively delayed as the concentration of sucrose was increased. In 0.88 M sucrose homogenates, the unbroken cells retained a normal appearance for hours. A possible explanation for the latter finding and for the preservation by concentrated sucrose solutions of mitochondria freed by cell rupture is that the intracellular osmotic pressure at the mitochondrial membrane may be considerably higher than the blood osmotic pressure.

15949

Blood Picture of Adult Gold Hamster (*Cricetus auratus*) After Castration.*

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It has been shown that gonadal hormones influence the blood picture in different species of vertebrates, the males having a higher number of corpuscles and greater hemoglobin values than the females. Steinglass *et al.*[1] showed a decrease in red blood count and hemoglobin value in castrated male rats; after testosterone administration the values return to normal. Vollmer *et al.*[2] claim that androgen raises the red blood count not only in castrated rats but in hypophysectomized and in normal animals.

Stein and Carrier[3] reported an appreciable drop (25 to 30% of the normal value) in the red blood cells of the "gold hamster" after castration, a decrease of red cell volume and an increase of mean corpuscular hemoglobin content, all of which disappeared after administration of iron, liver extract and testosterone propionate in periods as short as 3 days.

These results interested both the Hematology and Endocrinology Departments of our Institute and an investigation was planned to repeat this study.

Methods. Thirty-three adult male ham-

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* We are indebted to Mr. Guilherme Guinle for a grant for this investigation.

[1] Steinglass, P., Gordon, A. S., and Charipper, H. A., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 169.

[2] Vollmer, E. P., Gordon, A. S., Levenstein, I., and Charipper, H. A., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 409.

[3] Stein, K. F., and Carrier, E., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 313.

TABLE I.

Blood Picture of Normal Adult Gold Hamster. Average Values for 33 Male Animals.

R.B.C. 10^6 per cmm	Hb g per 100 cc	Vol. packed R.B.C. cc per 100 cc	M.C.V. (c.μ.)	M.C.H. (γγ)	M.C.H.C. (%)
8.1±0.22*	13.9±0.25	46±0.78	57±1.61	17.3±1.24	30±0.36

* Standard deviation of the mean.

TABLE II.

Blood Picture of Male Adult Gold Hamster.

Animal No.	Weight, g	Before castration R.B.C., 10^6 per cmm	Before castration Hb, g/100 cc	Before castration Vol. packed R.B.C. cc/100 cc	41 days after R.B.C., 10^6 per cmm	41 days after Hb, g/100 cc	41 days after Vol. packed R.B.C., cc/100 cc
2	90	8.3	15.2	48	8.4	14.4	49
3	95	8.3	15.3	49	7.3	13.8	48
6	115	7.9	13.8	51	8.3	14.0	42
7	134	7.4	15.2	53	8.7	15.2	43
8	140	6.7	15.4	53	9.0	14.0	49
	Avg	7.7±0.32	14.9±0.29	50±1.03	8.3±0.28	14.3±0.17	46±1.53

sters, weighing 76 to 145 g were used. Before and after castration red blood cell counts, hemoglobin and hematocrit determinations were made by technic previously described.[4] We emphasized that the hematocrit tube should be centrifugated for one whole hour at 3000 r.p.m. in order completely to settle the red blood cells of the hamster. 0.1 ml samples of blood from the heart were drawn each time and rendered incoagulable by the proper amount of a mixture of ammonium and potassium oxalate. We previously observed that this amount of blood could be drawn every 2 weeks without change in the blood picture of this mammal. In most cases cardiac puncture was done without anesthesia, in others after light ether anesthesia. Castration was performed by trans-scrotal route under avertina. Animals 15 to 19 were anesthetized by thionembutal (Abbott). Good results were obtained with intraperitoneal doses of avertina and thionembutal as used in the routine of our laboratories for rats.

Results. The normal hematological data for the male adult healthy hamster are shown in Table I. Our results are in reasonable agreement with those of Stein and Carrier.[3]

The first group of 5 castrated animals showed no alteration in blood picture after 41 days (Table II). The second group of 6 castrated showed no significant decrease 13 and 70 days after castration (Table III). The third group consisting of 12 castrated animals still showed normal blood picture 24 and 62 days after operation (Table IV); 5 normal controls were bled simultaneously with the castrated animals of this group, to check the maintenance conditions during the whole experimental period. As far as the blood picture is concerned the range of variation in both castrated and normal adult hamsters was not significantly different, according to the "t test" of Fisher.

We did not find the conspicuous alterations in the adult gold hamster after castration reported by Stein and Carrier;[3] we were interested only in observing the blood picture of the adult animals after castration. These authors emphasized that the blood picture is stabilized when the hamsters are 65-70 days old—"adult level"—so the effect of castration was observed only in 3 adult animals.

Summary. Thirty-three adult, healthy, male gold hamsters *(Cricetus auratus)* were used. Hematological data were obtained

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[4] Cruz, W. O., Martins da Silva, E., and Pimenta de Mello, R., *Mem. Inst. Oswaldo Cruz*, 1946, **42**, 609.

TABLE III.

Blood Picture of Male Adult Gold Hamster.

		Before castration			13 days after			70 days after		
Animal No.	Weight, g	R.B.C. 10^6 per cmm	Hb, g/100 cc	Vol. packed R.B.C. cc/100 cc	R.B.C., 10^6 per cmm	Hb, g/100 cc	Vol. packed R.B.C., cc/100 cc	R.B.C., 10^6 per cmm	Hb, g/100 cc	Vol. packed R.B.C., cc/100 cc
9	102	7.9	13.2	49	6.2	12.8	—*	—	—	—
10	115	9.4	14.6	47	7.5	11.0	—	8.6	16.6	51
11	145	9.6	17.4	54	7.7	13.0	—	6.3	14.0	45
12	100	8.8	14.8	47	6.7	11.2	—	6.2	13.0	44
13	110	9.2	15.4	48	5.8	9.0	—	5.7	11.4	41
14	95	8.7	14.6	44	6.2	11.6	—	5.4	11.2	40
	Avg	8.9±0.25	15.0±0.56	48±0.37	6.7±0.31	11.4±0.59		6.4±0.56	13.2±0.98	44±1.93

* Data accidentally lost.

TABLE IV.

Blood Picture of Male Adult Gold Hamster.

		Before castration			18-24 days after			60-62 days after		
Animal No.	Weight, g	R.B.C. 10^6 per cmm	Hb, g/100 cc	Vol. packed R.B.C. cc/100 cc	R.B.C., 10^6 per cmm	Hb, g/100 cc	Vol. packed R.B.C., cc/100 cc	R.B.C., 10^6 per cmm	Hb, g/100 cc	Vol. packed R.B.C., cc/100 cc
15	84	8.4	14.0	45	6.3	12.7	42	7.0	12.8	45
16	125	8.9	14.6	50	7.5	13.8	46	7.5	14.6	48
17	100	8.9	13.8	45	5.7	13.0	47	7.3	14.2	40
18	117	7.2	13.4	42	6.2	12.9	48	—	—	—
19	87	8.3	12.8	39	5.2	9.4	34	—	—	—
20	113	8.3	14.2	50	6.6	14.3	48	8.4	15.2	51
21	85	6.1	13.0	46	7.7	14.8	47	8.7	16.6	51
22	102	7.4	13.6	45	6.3	13.3	48	7.3	16.0	49
23	123	7.5	12.6	44	5.7	13.0	48	7.1	15.8	49
24	98	7.4	11.0	36	6.2	11.6	40	7.0	15.4	47
25	125	7.2	11.8	44	7.2	12.8	47	7.4	16.0	48
26	76	9.9	14.6	41	6.4	11.8	45	—	—	—
	Avg	7.9±0.29	13.3±1.00	44±1.17	6.4±0.67	12.8±1.27	45±1.24	7.5±0.20	15.0±0.38	47±1.12
					Values for 5 Normal Parallel Controls.					
		First sample			18 days after			60 days after		
35	92	6.2	13.2	46	8.1	13.0	37	7.0	13.2	48
36	139	8.9	11.6	41	6.0	12.7	45	8.2	14.0	50
37	101	7.1	12.0	38	4.7	9.5	42	7.0	12.6	47
38	118	7.1	13.4	43	7.3	12.4	46	—	—	—
39	105	10.6	14.8	46	8.0	14.0	54	8.6	15.8	51
	Avg	7.9±0.78	13.0±0.66	43±1.53	6.8±0.64	12.3±0.75	45±2.78	7.7±0.41	13.9±0.69	49±0.91

from the blood drawn by heart puncture before castration, and in 2 instances after castration. Castration failed to induce significant changes in the blood picture of 23 adult male hamsters.

15950

Hemoglobin and Glycemia Levels of the Adult White Mouse, Effect of Starvation and Central Depressants.

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A survey of the literature available has disclosed a dearth of published material on glycemia levels in mice. One group of workers[1] lists the blood sugar levels of hereditary dwarf mice as averaging 135 mg % (26 mice) and 139 mg % (16 mice). Others[2] give normal values between 76 and 86 mg %. Favour[3] has found values between 80 and 100 mg. Blood sugar levels of the commonly used albino rat seem to be no better known. One report[4] states that the blood sugar of (starved) rats is affected by age, season, and sex. These investigators determined blood sugar levels in 85 male albino rats averaging 114 g body weight, all of which were starved 17 hours previous to bleeding. The range of blood sugar levels extended from 100 to 148 mg %, the average being 113 mg. Myers and Bailey modification[5] of the Lewis and Benedict method was used for which 1 ml of blood was obtained by decapitation. Others[6] using the Folin-Wu method found an average of 122.2 mg % in 17 normal rats, the average of males being 4.7 mg higher than that of females.

Hemoglobin determinations in mice are likewise rare. In the chapter on histology[7] Fekete quotes from Hamm in Jaffé[8] stating that the hemoglobin content of mouse blood (based on the average of observations of 9 investigators) is 97% Sahli. Allowing 13 g hemoglobin per 100 ml of blood as 100% Sahli[9] this would be an average hemoglobin content of mouse blood of 12.6 g %.

Experimental. Adult albino mice of the Purdue Swiss strain were used. These were fed Purina Laboratory Chow in abundance such that extra pellets always remained in the cages. In this manner the mice were unstarved up to the time of bleeding. However, a more uniform level of glycemia can be obtained by starving the animals for a given period of time. Therefore our results of blood sugar determination in the nonstarved mouse appear higher than those reported elsewhere. Unless a standard procedure is followed by all, the results are not comparable.

Blood was obtained by sudden decapitation with a razor blade. We tried also bleeding from the tail with the mice wrapped in towelling to minimize struggling but with no apparent advantage. Blood hemoglobin was determined by the Sheard-Sanford method[10] using a photelometer[11] which was standardized by Wong's iron method.[12] The Sheard-Sanford method employs sodium carbonate to prevent acid hematin formation and thus eliminates a time factor correction. Blood sugar was determined by the Folin-Wu micro method[13] using 0.1 ml of blood, the photelometer being carefully calibrated with freshly prepared glucose solutions.

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[1] Marshak, A., Fernald, A. T., and Marble, A., *Am. J. Physiol.*, 1939, **125**, 457.

[2] Cammidge, P. J., and Howard, H. A. H., *J. Gen.*, 1926, **16**, 387.

[3] Favour, C. B., personal communication.

[4] Voegtlin, C., Dunn, E. R., and Thompson, J. W., *Pub. Health Rpts.*, 1924, **39**, 1935, Gov't. Prtg. Off. Wash. Rep. 943.

[5] Myers, V. C., and Bailey, C. V., *J. Biol. Chem.*, 1916, **24**, 147.

[6] Anderson, A. K., Honeywell, H. E., Santy, A. C., and Pedersen, S., *J. Biol. Chem.*, 1930, **86**, 157.

[7] *Biology of the Laboratory Mouse*, Blakiston, Phila., 1941, p. 92.

[8] Jaffé, R., *Anatomie und Pathologie der Spontanerkrankungen der kleinen Laboratoriumstiere*, Julius Springer, Berlin, 1931.

[9] Best, C. H., and Taylor, N. B., *The Physiological Basis of Medical Practice*, Williams & Wilkins, Balt., 4th ed., p. 43.

Because the control blood sugar readings seemed abnormally high we suspected a factor of excitement causing sympatheticomimetic hyperglycemia. In order to produce sedation we tried exposing mice to chloroform vapor until quiet. This resulted in no lowering of glycemia so next we administered sodium pentobarbital (nembutal) intraperitoneally. Because of the reduction of blood sugar to less than half that of the controls we suspected an abnormal hypoglycemia and compared the effects of other central depressants namely: diallyl barbituric acid (dial), sodium ethyl (*l*-methyl-butyl) thiobarbiturate (pentothal), and chloral hydrate, all of which were administered intraperitoneally. Other groups of mice were subjected to fasting and blood sugar determined at periods of 12, 18 and 24 hours.

Results and Discussion. Results are shown in Tables I, II, and III. Our findings for hemoglobin in mice were somewhat higher than those reported by Jaffé. According to the Sahli scale ours would average 107% where Jaffé listed 97% Sahli. Environmental and strain differences would be significant in causing differences in blood chemistry. By way of comparison we have listed in Table I hemoglobin values from 48 white rats used in another study. A close similarity can be seen in the 2 species. Table II shows the effect of fasting on the glycemia level.

TABLE I.
Comparison of Hemoglobin in White Rats (Wistar) and White Mice (Purdue Swiss).

No. animals used	Hb g % (avg)	S.E.
48 rats	13.1	0.7
86 mice	13.9	0.26

TABLE II.
Effect of Starvation up to 24 Hours on Blood Sugar Level of White Mice (Purdue Swiss).

	No. mice used	Blood sugar mg % avg	S.E.
Controls (unstarved)	61	173.8	5.9
Starved 12 hr	12	125.6	7.7
″ 18 ″	12	102.8	7.6
″ 24 ″	22	108.9	3.6

Fasting causes a fall in glycemia level as would be expected, a greater drop occurring in the first 12-hour period than in the second. A rise in blood sugar occurs after 18 hours which is probably due to gluconeogenesis at this period. We have noted in other studies with rats that fasting causes the greatest drop in blood sugar level during the first 10 hours with a slower falling subsequently.

Table III shows the effects of depressant substances on the blood sugar level of nonfasted mice. The nonfasting level of glycemia seems high but possibly is characteristic for mice in the presence of abundance of food. Hyperglycemia has been shown to be a recessive character in mice;[2] hypoglycemia as a hereditary condition is also known.[14] The hyperglycemia we obtained after injection of dial and chloral hydrate may possibly have been due to excitement and the lack of anticonvulsive action of the drugs. Evidence for this lies in the fact that when the mice became prostrate, sounds like snapping of the fingers would startle them excessively. Possibly the loss of voluntary action occurred simultaneously with increased afferent excitability. Recently[15] it has been shown that different members of the barbiturates vary in hypnotic and anticonvulsive activity even in doses causing sedation and ataxia. That

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[10] Sheard, C., and Sanford, A. H., *J. Lab. and Clin. Med.*, 1929, **14**, 558.

[11] Sanford, A. H., Sheard, C., and Osterberg, A. E., *Am. J. Clin. Path.*, 1933, **3**, 405.

[12] Wong, S. Y., *J. Biol. Chem.*, 1928, **77**, 409.

[13] Folin, O., and Wu, H., *J. Biol. Chem.*, 1928, **77**, 421.

[14] Cammidge, P. J., and Howard, H. A. H., *Proc. Roy. Soc. Med.*, 1930, **23**, 1341.

[15] Everett, G. M., and Richards, R. K., *Fed Proc.*, 1945, **4**, 20.

TABLE III.
Effect of Depressant Drugs on Blood Sugar Level of White Mice (Purdue Swiss).

	No. mice used	Dosage mg/kg	Blood sugar mg % (avg)	S.E.
Controls	61	—	173.8	5.9
Chloroform	12	vapor	172.9	4.8
Nembutal	24	100	70.1	3.5
Dial	12	50	208.0	3.6
Chloral hydrate	11	200	195.3	6.6
Na-pentothal	12	75	172.8	4.2

nembutal caused such a marked hypoglycemia is difficult to explain with our present knowledge. First one group of 12 mice was injected with nembutal and when the hypoglycemic action was noted a second group of 12 (at a later date) was injected in a similar fashion. The 2 groups showed identical responses. The effect of nembutal on glycemia level has been noted by others[16] in mice and rats, and even in fasted rats.

Summary. Hemoglobin in 86 and blood sugar levels in 61 nonstarved albino mice of the Swiss strain were determined. The average hemoglobin for the mice is 13.9 g per 100 ml of blood. Blood sugar averaged 173.8 mg per 100 ml of blood in nonfasted mice falling to 108.9 mg in 24 hours. A slight rise occurred between 18 and 24 hours. The effects of 5 central depressants were investigated, the only one of which causing a drop in blood sugar being nembutal.

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[16] Long, C. N. H., personal communication.

15951

Antibacterial Lipids from *Tetrahymena geleii.*

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It is well known that many species of protozoa ingest and destroy myriads of bacteria. Metchnikoff[1] was probably the first to point out the similarity between phagocytosis in higher animals and intracellular digestion by protozoa, although in neither case is the mechanism clearly understood. Opie[2] has demonstrated the presence of proteolytic enzymes in phagocytes but that they are responsible for the killing of bacteria has not been shown. The presence of antibacterial substances within the phagocyte has been postulated but attempts to extract such substances have, in general, yielded preparations of only weak bactericidal or bacteriostatic properties.[3,*] It seems possible that the bactericidal effect of the living phagocyte may be due to conditions within the cell unfavorable to bacteria such as low pH rather than to the presence of a specific antibacterial agent. Although the same may also be true for the protozoa it was decided to test this point by attempting the isolation of sub-

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[1] Metchnikoff, E., *L'immunité dans les maladies infectieuses*, Paris, 1901.

[2] Opie, E. L., *J. Exp. Med.*, 1945, **8**, 410.

[3] Rich, A. R., *The Pathogenesis of Tuberculosis*, p. 289, Chas. C. Thomas, Springfield, Ill., 1944.

[*] Nutini and Kreke (*J. Bact.*, 1942, **44**, 661), and Nutini and Lynch (*J. Exp. Med.*, 1946, **84**, 247), have prepared from various animal tissues, especially spleen and brain, substances having antibacterial properties active both *in vitro* and *in vivo*.

TABLE I.
Antibacterial Activity of Lysates of Four Species of Protozoa.

Organism	Antibacterial activity*				
	Staph "H"	*E. coli*	*Klebsiella pneumoniae*	*M. phlei*	*M. tuberculosis* "H37Rv"
Astasia klebseii	0	0	0	0	
Chilomonas paramecium	0	0	0	0	
Euglena gracilis	0	0	0	0	
Tetrahymena geleii W	0	0	0	1/40	1/10
" " H	0	0	0	1/8	1/8
" " GHH	0	0	0	1/8	1/8
" " E	0	0	0	1/10	
" *vorax* V	0	0	0	0	
" " PP	0	0	0	0	

* Activity is expressed in terms of highest dilution inhibiting growth.

TABLE II.
Bacterial Spectrum of Three Active Preparations.

Test organism	Active preparation				
	W7	W8	(Solids determination showed 23.6 mg/ml)	W9-10	(Solids determination showed 28 mg/ml)
Mycobacterium tuberculosis "H37Rv"	1/300*	1/700*	33.7†	1/860*	32.6†
" *phlei*	1/200			1/667	42.
Pneumococcus Type I	1/6800	1/8000	2.95	1/16000	1.13
" " III	1/9500	1/8000	2.95	1/16000	1.13
Streptococcus viridans	1/2500	1/4000	5.9	1/4000	4.5
" *pyogenes* "C-203"	1/1220	1/2000	11.8	1/2000	9.
Staphylococcus "H"	1/54	<1/10		1/154	182.
Escherichia coli	<1/10	<1/10		<1/10	
Salmonella enteritidis	<1/10	<1/10		<1/10	
Shigella dysenteriae Shiga	<1/10	<1/10		<1/10	

* Highest dilution inhibiting growth of test organism.
† Least amount in μg/ml inhibiting growth of test organism.

stances having antibacterial properties.

Bacteria-free cultures of *Astasia klebseii, Chilomonas paramecium, Euglena gracilis,* and *Tetrahymena geleii,* were obtained through the courtesy of Professor George W. Kidder, Amherst, Mass. In preliminary experiments the organisms, grown in a tryptone-acetate or a proteose-peptone broth, were sedimented in the cold, resuspended in 1/10 volume of the clear supernate, and repeatedly frozen and thawed. These lysates were then tested for inhibitory activity against *Staphylococcus aureus, E. coli, Klebsiella pneumoniae* and, in certain cases, 2 strains of mycobacteria, one a virulent human strain and the other a saprophyte.

It will be seen from the data presented in Table I that growth of both *Mycobacterium tuberculosis* and *M. phlei* was inhibited by lysates of *Tetrahymena geleii* but not by lysates made from the other strains. Filtration of these lysates through Seitz filters removed all activity. None of the lysates inhibited growth of the other 3 bacterial cultures tested. The same lysates when tested against a culture of *Photobacterium fischeri* showed no antiluminescent activity.

Since growth of both *M. tuberculosis* and *M. phlei* was inhibited by lysates of *Tetrahymena geleii,* it became of interest to obtain, if possible, preparations of greater purity and potency for more extensive study. For this purpose, Blake bottles containing 200 ml of 1.5% proteose-peptone broth were inoculated with 4 ml of a 24-hour culture of *Tetrahymena geleii* "W" and incubated horizontally for 4-5 days at 25°C. The organisms were then sedimented in a Sharples centrifuge and stored in the frozen state until used. The yield of moist sedimented organisms was approximately 1.3%.

Preliminary experiments showed that organic solvents (ether, acetone, and methanol) extracted the active material from the lysed organisms and that activity could be re-extracted from the ether solution with aqueous alkaline solutions. Details of extraction will be given in a later section.

Experiments *in vitro* and *in vivo* which have been carried out with various active fractions are presented below.

Activity in vitro. Three solutions which were prepared from sedimented whole organisms in the following manner were tested *in vitro* against a variety of organisms: (1) W7 was a 1% Na_2CO_3 extract of ether soluble material, (2) W8, a water solution of the precipitated sodium salts obtained from an extract of hexane soluble material, and (3) W9-10, a NaOH extract of ether soluble material. A broth dilution test was used. Kirchner's synthetic medium with the addition of Tween 80[4,5] to induce diffuse growth was used for tests with acid-fast organisms. Tests with other organisms were made in a beef heart infusion broth. Both media were well buffered and results were not affected by the alkaline nature of the solutions tested. The cultures used in testing were diluted as follows: acid-fast organisms 1-50, pneumococcus and streptococcus cultures 1-100, and staphylococcus and Gram-negative cultures 10^{-6}. The diluted cultures were dispensed in 13 x 100 mm tubes in 2 ml amounts and the inhibiting agents added with a Kahn pipette in decreasing amounts; usually from 0.1 to 0.01 ml. The incubation temperature was 37°C. Tests with *M. phlei* and *M. tuberculosis* "H37Rv" were read after 3 and 7 days respectively. Other tests were read after 18 hours. The results of these tests are given in Table II.

All preparations showed high activity against the pneumococcus and the streptococcus with a lesser activity against the acid-fast cultures. Two of the preparations showed slight activity against the staphylococcus, but all were inactive against the Gram-negative bacilli.

TABLE III.
Effect of Serum and Blood on Activity *in Vitro.*

Preparation	B.H.B.	B.H.B. 5% serum	B.H.B. 5% blood
W9-10	1.5	>240	>240
W11-12	3.2	90	760
Py7-10-11D	18.5	>250	

Possible inhibition by serum and blood of activity *in vitro* against the Type III pneumococcus was tested. The medium used was a beef heart infusion broth unenriched and with the addition of either 5% horse serum or 5% rabbit defibrinated blood. All of the 3 preparations tested showed greatly reduced activity in the presence of serum or blood. The results given in Table III show the minimum inhibiting concentration in micrograms per ml.

The active preparations described above were all obtained from disrupted protozoa. The culture medium after removal of the protozoa showed no antibacterial activity. However, 2 supernates after Sharples centrifugation were acidified to pH 2.5, extracted with chloroform and the chloroform re-extracted with a small volume of sodium carbonate solution. One such extract (95 ml) obtained from 16 liters showed activity in a dilution of 1-54 against *M. phlei* and another extract (30 ml) obtained from 17½ liters was active in a 1-70 dilution. Since Sharples centrifugation did not remove all the organisms from the culture medium, the slight activity obtained probably was derived from the organisms still remaining in the culture medium.

In the early experiments in which lysates of several species of protozoa were tested for antibacterial activity only those from *Tetrahymena geleii* showed activity. Since preparations of much higher potency were later obtained from *Tetrahymena geleii* by the extraction methods described, the species *Chilomonas paramecium* was subjected to the latter procedure. An extract so prepared proved to be highly active when tested against the Type III pneumococcus. Work with this extract was not continued, however, since it appeared that the active material was similar

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[4] Dubos, R. J., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 361.

[5] Dubos, R. J., and Davis, B. D., *J. Exp. Med.*, 1946, **83**, 409.

TABLE IV.
Toxicity for Mice of Preparation W9-10.

Dose, mg	Route of inj.	Lived	Died
14	Intraperitoneal	5	0
14	Subcutaneous	5	0
7	Intravenous	0	10
3.5	”	6	4
1.75	”	5	0

in nature to that from *Tetrahymena gelei.*

Toxicity. Preliminary tests on toxicity for mice of preparations W9-10 were made. The results are given in Table IV.

Peritonitis developed in those mice injected intraperitoneally, and cellular infiltration and necrosis of the skin in those injected subcutaneously.

Since there were indications at this point that the substances under investigation were fatty acids, more extensive toxicity studies were deemed unnecessary.

Activity in vivo. Although both serum and blood decreased activity *in vitro* indicating probable inactivity *in vivo,* protection test in mice against *M. tuberculosis* "H37Rv" were carried out with 2 preparations, Py6 and Py7. The minimum inhibiting concentrations of the 2 preparations against *M. phlei* were respectively 25.6 and 73.8 μg/ml. Treatment, which was begun immediately after infection, consisted of the subcutaneous injection of 5 mg per day.

Suspensions of culture made from surface growth on Kirchner's medium were injected intravenously into white mice weighing 17 g. In the first experiment a light infecting dose was used (0.5 ml of a filtrate through Whatman No. 1 filter paper of a 1 mg/ml suspension). Treatment was continued for 26 days. In the second experiment the infecting dose was heavier (0.5 ml of a 1 mg/ml suspension) and treatment was continued for 13 days. Each experiment consisted of 3 groups of 10 mice, one group treated with one of the above preparations, one with streptomycin (5000 units per day given in 3 daily doses), and one untreated group. The mice were sacrificed immediately after treatment was stopped and the extent of lung and spleen lesions noted. In both experiments the mice treated with Py6 and Py7 showed as extensive lesions as the controls while infection in the group treated with streptomycin was retarded.

Inasmuch as all preparations showed high activity *in vitro* against the Type III pneumococcus protection tests in mice against this organism were also performed.

Mice were infected intraperitoneally with 1 ml of a 10^{-6} dilution of a 6-hour blood broth culture. Three partially purified preparations were tested W9-10, Py6 and Py7. Three subcutaneous injections of 5 mg each were given, one immediately after infection and 2 on the following day. No protection was afforded. All treated mice died as quickly as controls.

Chemical Fractionation. In view of the marked *in vitro* activity of these various crude preparations, fractionation experiments were undertaken in an effort to isolate in pure form and identify the active principle.

The following procedure is that eventually devised for the isolation and purification of this acidic material. Acetone was found to be the solvent of choice for the quantitative extraction of the active material from the disrupted organisms. In a typical run, 568 g of the moist organisms were mixed in a Waring Blender with 250 ml of acetone and the brei then centrifuged. This procedure was repeated with 4 more 250 ml portions of acetone. The dried acetone-insoluble material weighed 16.5 g and no longer yielded active material on extraction with various solvents. The combined acetone extracts were concentrated *in vacuo* to remove the acetone and the remaining cloudy aqueous solution was continuously extracted with ether for 24 hours. The aqueous phase, after removal of the ether, showed no inhibiting activity and was discarded. Preliminary experiments had shown that the entire activity could be extracted from the ether solution with 1% sodium hydroxide solution, but with the aim of effecting a fractionation of the acidic materials, preliminary extraction with 1% sodium carbonate solution was carried out.

Ten 25 ml portions of 1% sodium carbonate were used, the extracts combined and reextracted with ether following acidification. The ether solution upon evaporation yielded an oily residue weighing 3.1 g. Made up in solution in 1% sodium carbonate at 5 mg per ml this fraction inhibited *M. phlei* at 1 to 68 dilution.

The original ether solution remaining after the extraction with 1% sodium carbonate was extracted with four 25 ml portions of 1% sodium hydroxide solution. These were combined, acidified and reextracted into ether from which was obtained on evaporation a pigmented, oily residue weighing 890 mg. The original sodium hydroxide solution, which therefore contained 8.9 mg per ml, had shown inhibition of *M. phlei* at 1 to 280 dilution.

The ether solution remaining after the 1% sodium hydroxide extraction was washed with water, dried and evaporated. A semicrystalline residue weighing 1.288 g remained. This fraction showed no inhibiting activity. Crystallization of this material from absolute alcohol yielded 370 mg of glistening platelets of a neutral compound which melted at 309 to 312°. It contained no nitrogen or sulfur and analyzed for a compound of the formula $C_{27}H_{45}OH$, isomeric with cholesterol. The mother liquors left an oily residue which probably consisted of neutral lipids.

The 3.1 g from the sodium carbonate extract were taken up in acetone and the solution centrifuged to remove 108 mg of insoluble material. After treatment with Norite and concentration, the acetone solution deposited several crops of colorless leaflets which were combined and recrystallized from acetone. From 700 mg of crude crystals melting from 49 to 53° there were obtained 150 mg of colorless needles melting at 53-54°. The mother liquors yielded further material of slightly lower melting point. In the original acetone mother liquors there remained 2.4 g of oily acidic material.

The crystalline material of melting point 53-54° contained no sulfur or nitrogen and yielded the following analytical values.

Found: C, 73.85; H,12.60
Calcd. for $C_{13}H_{27}COOH$: C, 73.63; H, 12.36

The neutralization equivalent was somewhat higher than that required for myristic acid indicating that this product may still be a mixture of fatty acids. However, there appears to be little doubt but that this C_{14} fatty acid is the chief component of this fraction.

By the same treatment with acetone, crystalline material was also obtained from the acidic residue obtained by sodium hydroxide extraction of the ether solution. The top fraction of crystals weighed 188 mg and melted at 50-52°; it appeared to be identical in every regard with that previously obtained. It thus appears that no fundamental fractionation was achieved by the sequence of alkaline solutions, and in subsequent preparations the use of sodium carbonate extraction was abandoned.

When the crystalline fatty acid, fraction Py9 (chiefly myristic), was tested for inhibitory activity, it yielded the results shown in Table V. Other purified fatty acids, palmitic, stearic and oleic, were tested for comparison of activity with that of the material from *Tetrahymena geleii*. The results are also shown in Table V. The inspection of these data disclosed a significant qualitative and quantitative difference between the activities of the purified material and the activities of the crude fractions. A comparison of the activities of the saturated fatty acids with those of oleic acid showed the same sort of variations and it became of interest therefore to determine roughly the proportion of saturated to unsaturated fatty acids in the material from *Tetrahymena geleii*. The presence of these latter acids might account for the higher activity of the crude oily fractions. The neutralization equivalent of a pooled sample of crude acids from *Tetrahymena geleii* was determined and found to be 350, indicating an average chain length of 22-24 carbon atoms. A neutral solution of the acid mixture was treated with lead acetate solution to precipitate the fatty acids, whereupon the dried lead salts were separated into ether soluble and ether insoluble portions. The weights of the acids obtained by decomposing the lead salts showed a distribution of 45% saturated fatty acids and 55%

TABLE V.
Bacterial Spectrum. Crude and Purified Preparations Compared with Oleic, Stearic and Palmitic Acids.

Preparation	*M. tuberculosis* "H37Rv"	*M. phlei*	*M. smegmatis*	Pneumococcus Type III	Streptococcus "C-203"	Staphylococcus
W11-12, crude	29.5	70.7	137.4	3.4	19.1	8.2
Py7-10-11D, crude	65.	375.	375.	18.5	80.6	160.
Py14 saturated fatty acids	80.	303.7	405.	11.8	160.	
Py15 unsaturated fatty acids	80.	185.	217.5	8.5	32.5	240.
Py9 crystalline (myristic acid)	127.5	>500.	>500.	5.0	>500.	240.
Oleic acid	46.7	88.	187.	1.4	14.8	15.
Stearic acid	46.3	>500.	>500.	6.5	>1000	
Palmitic acid	33.4	>500.	>500.	6.5	>1000.	

The figures above represent the minimum inhibiting concentration in micrograms per ml. Py14 and Py15 were derived from crude preparation Py7-10-11D. The activity of some of the above preparations was difficult to determine. Py9, stearic and palmitic acids rendered the test medium so turbid that it was difficult to distinguish between turbidity due to the preparation or that caused by growth of the test organism.

unsaturated fatty acids. The inhibitory activity of these fractions, Py14 and Py15 respectively, is given in Table V.

The iodine number of the crude acid fraction, as determined by Hanus' method, was found to be 35. This value corresponds to a mixture of approximately equal amounts of saturated acids and unsaturated acids, assuming an iodine number of about 75 for the latter.

In a recent paper Dubos[6] states that all fatty acids which he has tested exerted a bacteriostatic effect on tubercle bacilli in a protein-free medium, and that unsaturated fatty acids had the most pronounced effect. He further states that this toxicity can be abolished either by esterification or by addition of crystalline serum albumin. Since the medium we have used for testing inhibition of growth of acid-fast organisms by fatty acids contains 0.05% serum albumin, it is probable that a higher degree of inhibition would have been obtained in a medium without serum albumin.

Discussion. Very little information is available concerning the chemical constituents of protozoa and no reports whatever have been found dealing with the lipids of these microorganisms. Our findings are, therefore, of interest in that they augment the general survey of plant and animal lipids so adequately presented by Hilditch in 1940.[7] While no thorough and systematic fractionation of the lipid material from *Tetrahymena geleii* was made, this fraction of the cellular components could be characterized as follows:

1. The lipid fraction represents from 15 to 20% of the cellular solids.
2. Of this fraction nearly 75% is free fatty acids with only 15% as neutral fat and the remainder a neutral steroid-like compound.
3. The fatty acid fraction represents a complex mixture of saturated and unsaturated acids from which myristic acid has been crystallized. Titration values indicate a preponderance of longer chain acids with an average length of 22 carbon atoms.

These characteristics are in general agreement with observations on other microorganisms; the lipids of the tubercle bacillus, according to Anderson,[8] "consist chiefly of a complex mixture of *free* fatty acids, with relatively small amounts of neutral fat;" and, quoting Hilditch,[7] "the fats of the simplest and most primitive organisms are usually made up from a very complex mixture of fatty acids. Palmitic, myristic and stearic acid are often present in fats of aquatic origin, as also may be unsaturated C_{14} and even C_{24} acids."

The observations reported in this paper concerning the bacteriostatic activity of these fatty acids are in agreement with other similar observations recorded in the literature.[9–12] The failure to obtain protection *in vivo* against infections by organisms which are very susceptible *in vitro* to the action of these agents has also been the common finding.[13]

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[6] Dubos, R. J., *J. Exp. Med.*, 1947, **85**, 9.

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The bactericidal activity of the fatty acids and, since they are usually tested in alkaline or neutral media, their salts, has been recognized as primarily a function of their surface tension lowering capacity[14] with no great specificity attending their molecular structure.[15,16] In this regard they behave similarly to the synthetic detergents[17] and to some of the more recently discovered antibiotics such as subtilin, tyrocidin and gramicidin.[18,19] The explanation of their almost negligible activity *in vivo* appears to lie in their ready absorption by proteins[5,6,20] or through complex formation with phospholipids.[21]

Summary. 1. A mixture of fatty acids which inhibited the growth of mycobacteria was obtained from lysed cultures of *Tetrahymena geleii.* 2. This material could be extracted with organic solvents from the disrupted organisms and purified by reextraction into dilute alkaline solution. 3. The fatty acids in the mixture had an average chain length of 22-24 carbon atoms and represented an approximately 50/50 mixture of saturated and unsaturated acids. Myristic acid was isolated in crystalline form. 4. Partially purified preparations were active *in vitro* against pneumococci, streptococci, mycobacteria and staphylococci, but not against Gram-negative bacilli. Activity was greatly reduced in the presence of blood or serum. 5. No activity *in vivo* against infection of mice with mycobacteria or pneumococci could be demonstrated.

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15952

The Effect of Sodium Fluoride on the Metabolism of Oral Lactobacilli.*

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(Introduced by L. P. Gebhardt.)

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Dental caries has been shown to be associated with high lactobacillus counts in saliva by the investigations of Jay,[1] Dean and his associates,[2] Snyder,[3] Collins *et al.*,[4] and others.[5] Almost all investigators now agree that the number of lactobacilli in the saliva is a rough index of caries activity.

Children of school age in the areas in which the drinking water contains 1 p.p.m. or more of fluoride have a low caries incidence. Dean[6] found that the lactobacillus counts in such areas were lower than in areas in which the water contained no fluoride. More recently,[7,8] the use of a solution of NaF as a mouth wash decreased the number of lactobacilli. Fosdick and Starke,[9] have shown that *Lactobacillus acidophilus* will degrade glucose according to the Meyerhof scheme to lactic, pyruvic, and phosphoglyceric acids. Any of these acids are capable of dissolving enamel. Sodium fluoride has been shown to inhibit the formation of acid from glucose by lactobacilli in concentrations as low as 2 p.p.m.[10] Concentrations from 2 to 100 p.p.m. did not inhibit growth.

There has as yet been no complete explanation of how fluoride water inhibits caries or of how the fluoride inhibits the activities of oral lactobacilli.[11] Some information should be secured by studies of its effect on specific enzymes and of the effect of continued growth in the presence of fluoride. With this in mind an investigation was made of (1) the effect of NaF on the acidogenic properties of several strains of lactobacilli from saliva, (2) the acidogenic properties of a lactobacillus grown in the presence of NaF for 6 months, and (3) the effect of NaF on the activity of 9 dehydrogenases of a lactobacillus.

Effect of Added NaF on Acid Production in Glucose Broth. Eleven strains of lactobacilli were isolated from saliva and maintained in peptonized milk agar. Five-tenths ml of a 48-hour tryptose phosphate broth culture of these organisms was planted in flasks containing 100 ml of tryptose phosphate broth with .5% glucose. Sodium fluoride was added to some of the flasks to make a concentration of 1 p.p.m. and to others to make a concentration of 100 p.p.m. The acid was titrated at intervals with N/10 NaOH. The titratable acidity of duplicate flasks never varied more than 2 ml of N/10 acid per 100 ml medium.

Table I shows that NaF in concentrations of 1 p.p.m. and 100 p.p.m. inhibit acid formation from glucose at all stages investigated in the growth of the lactobacilli. The acid production was inhibited to some extent for

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TABLE I.
Average Reduction in Acid by NaF. Eleven Strains of Lactobacilli.

	Reduction per 100 ml medium	Hours incubated			
		24	48	72	96
1 p.p.m. NaF	ml N/10 acid	3.3	6.2	6.8	9.2
	% reduction	8.8	10.9	10.5	12.6
100 p.p.m. NaF	ml N/10 acid	4.4	12.5	16.5	20.2
	% reduction	11.4	21.5	25.0	27.3

each organism. The smallest reduction was shown in the first 24-hour period, and the greatest reduction in the 96-hour period. The higher concentration of the fluoride was roughly twice as effective as the lower in preventing acid formation. Growth of the organisms was not decreased by either concentration of fluoride as far as could be determined by visual observation of turbidity.

Effect on Acid Production of Continued Growth in the Presence of NaF. A single strain of lactobacillus, Strain 1, isolated from the saliva of an individual with rampant caries was grown in peptonized milk agar containing 100 p.p.m., 1 p.p.m., and no NaF and was maintained by weekly transfers over a period of 6 months on these media. This organism, Strain 1, was a strong acid producer. At the end of 6 months, organisms from the plain peptonized milk agar, organisms from the agar containing 1 p.p.m. NaF, and those from agar containing 100 p.p.m. NaF were inoculated into tryptose phosphate broth containing .5% glucose. The acid was titrated as before.

Continued growth in the presence of 1 p.p.m. NaF had no effect on the acidogenic properties of the strain used, but in the presence of 100 p.p.m. NaF, the organism lost 53% of its ability to produce acid. Repeated titrations showed little or no variation. To check this result the original Strain 1 was again grown for 6 months, in a medium containing 100 p.p.m. NaF. It again showed a marked loss of ability to produce acid when planted in broth with no NaF present. Maintenance of this trained strain on plain peptonized milk agar for 6 months did not cause it to regain its acidogenic properties. Gram stains showed no morphological changes.

Effect of NaF on Dehydrogenase Activity. The Thunberg technic was used. Two control tubes containing the substrate but no NaF, 2 with substrate and 1 p.p.m. NaF, and 2 with substrate and 100 p.p.m. NaF were set up simultaneously. The lactobacilli were obtained by growing in 100 ml of tryptose phosphate broth for 48 hours, centrifuging, washing once with distilled water, and suspending in distilled water. The suspension was adjusted to a standard turbidity (500 on a Kletts-Summerson photoelectric colorimeter) and was used immediately since storage in the refrigerator seemed to decrease the dehydrogenase activity. Light suspensions were relatively inactive. Slight variations from the above standard density produced only small changes in activity. The organisms were placed in the side arm of the tube and tipped in after the tubes had reached a temperature of 37°C. The time was noted for the decolorization of the control tubes and the increase or decrease in time for the tubes containing NaF. The time of decolorization of the control tubes was given a value of 100, while values for those with NaF were calculated as percentages of activity of the controls. A tube requiring twice as much time for decolorization as that of the control was given a value of 50 as its dehydrogenase activity. The results are shown in Table II. One hundred p.p.m. NaF inhibited all but 1 of 9 dehydrogenases but 1 p.p.m. imparted no inhibitory, slight inhibitory, or even stimulating action.

Discussion. As little as 1 p.p.m. NaF decreased acid production from glucose. This inhibition may explain the low caries incidence in those areas in which the water supply contains fluoride. There was considerable variation among the strains in their susceptibility to fluoride, 4 showing no signi-

TABLE II.
Effect of 1 p.p.m. and 100 p.p.m. NaF on the Dehydrogenase Activity of Strain 1.

Substrate	1 p.p.m.	100 p.p.m.
Glucose	87*	60
Levulose	100	80
Maltose	100	71
Sucrose	100	0
Lactose	140	100
Glycerol	100	0
Pyruvic acid	95	43
Lactic acid	94	89
Formic acid	180	25

* Values are expressed as percentages of activity of the control which had no added NaF.

ficant decrease with 1 p.p.m., although 100 p.p.m. did inhibit acid production. Less inhibition was found in the first 24 hours than in the later periods of growth. Bibby and Van Kestern[10] working with 12 strains of lactobacilli from healthy mouths obtained values similar to those found in this work.

Continued growth of a strain of lactobacillus in the presence of 100 p.p.m. NaF decreased its ability to produce acid from glucose. This suggests that an alternate enzyme system is used. Wiggert and Werkman[11] obtained fluoride resistant strains of *Propionibacterium pentosaceum* by growing the organism in a medium containing fluoride. These strains were incapable of fermenting phosphoglyceric acid. They suggested that this organism was capable of fermenting glucose in the presence of fluoride by a route other than that involving phosphoglyceric acid. Nord and Mull[12] point out that in the fermentation by *Fusaria*, phosphoglyceric acid was neither isolated in the presence of fluoride nor utilized as a carbon source. The strain of lactobacillus reported in this paper, Strain 1, which was grown in media containing fluoride, may have a similar metabolism. Such a change in metabolism may occur in lactobacilli in plaques on the teeth. The plaque being repeatedly exposed to small concentrations of fluoride in drinking water may adsorb sufficient quantities to bring about such changes in the organisms with which they are in contact. NaF, then, may reduce decay by the prevention of acid formation from fermentation by lactobacilli in the mouth. This acid production may be reduced in one or both of 2 ways. First, by the direct inhibition of the enzymes necessary to the formation of acid and second, by altering the enzymatic pattern of the organism by being constantly present in the environment.

That NaF is capable of affecting the oxidative mechanisms of the lactobacillus is shown in the results of the dehydrogenase determinations. The substrates were chosen because they were carbohydrates likely to be encountered in the mouth by lactobacilli, or were possible intermediates in the breakdown of sugars. Previous experimental work in this laboratory had shown that the activities of the dehydrogenases for these substrates were remarkably constant for 5 strains that were tried. No dehydrogenase activity was shown for either succinic acid or acetic acid. The inhibition of oxidation of formic, lactic and pyruvic acids would appear to have no relation to decreased acid production from glucose since oxidation of these acids would lead to less accumulation of acid. Preventing the oxidation of glycerol if it is formed, would prevent acid formation. The point of inhibition of acid formation of lactobacilli from glucose would seem to lie in the inactivation of an enzyme involved in changes before phosphoglyceric acid is formed. The dehydrogenase for glucose is inactivated by both concentrations of fluoride.

Just what part is played in the prevention of caries by the action of NaF on the dehydrogenases for sucrose, maltose, and lactose is impossible to determine from this experiment. It may be of no significance, since all of the above carbohydrates are hydrolyzed to glucose in the normal mouth. One hundred parts per million of NaF prevents acid formation from lactose but does not inactivate the dehydrogenase for lactose. The inability to inactivate a carbohydrate dehydrogenase does not always indicate inability to prevent acid formation.

Summary. 1. Of 11 strains of lactobacilli tested, all were reduced in their ability to produce acid by the addition of 1 and 100 p.p.m. of NaF. Resistance to the action of fluoride on acid production varied among strains.

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[12] Nord, F. F., and Mull, R. P., *Advances in Enzymology, Vol. V., Interscience,* New York, 1945, p. 165.

2. Continued exposure to 100 p.p.m. NaF lowered the ability of an actively acidogenic strain of lactobacilli to produce acid from glucose. This reduced acidogenic activity continued even in the absence of NaF. One p.p.m. of NaF had no demonstrable effect.

3. One hundred p.p.m. NaF inhibited the dehydrogenases of lactobacilli for 8 out of 9 substrates. The inhibition by 1 p.p.m. was much less.

15953

Effect of the Fat Content of Diets on Blood Sugar.

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The adverse effect of fat feeding on the carbohydrate tolerance of normal persons has been amply demonstrated. Staub[1] and Kageura[2] 25 years ago discovered the fact that healthy men, after being kept on carbohydrate-free meals for 2-3 days, responded to glucose feeding with "diabetic-like" blood sugar-time curves and glycosuria. Himsworth[3] obtained identical results with high fat diets even when including carbohydrates not in excess of 50 g per day. In these studies no attention was paid to the postabsorptive blood sugar level, but the data in the reports of these authors disclosed no rise above the normal level.

Changes in the postabsorptive glycemic level after fat feeding were first reported by F. M. Allen and his associates.[4] Their observations were made on epileptic persons who were fed 260 to 580 g of fat daily, with only 12 to 22 g of protein and 2.5 to 3.7 g carbohydrate. Periodically performed determinations of the plasma sugar showed marked and gradual increases, as for instance (in the most responsive subject) from the initial 135 mg % to 357 mg % in 7 weeks.

Allen's observations, and a number of other studies related to the subject, were carried out under rather extreme experimental conditions. Our interest lay in the effect of high fat diets which, at the time of our studies here reported, were (and very widely still are) prescribed to diabetic patients. Accordingly, the carbohydrate ration was 100-150 g, daily, with unrestricted protein consumption. We selected for the experiment healthy persons, whose carbohydrate tolerance was normal and whose eating habits fitted into a pattern which can be considered as representing the normal, average type. They customarily consumed 80-120 g protein, 80-120 g fat, and 200-300 g carbohydrate. For the experiment the protein was kept at the usual level, the fat was increased to 200-250 g, and the carbohydrate was limited to approximately 100 g. Just prior to changing to the high fat diet the sugar content of the venous blood was determined in the postabsorptive state. In most instances several determinations were made on consecutive days while the subjects were still on the normal diet in order to ascertain the extent of normal fluctuations of the fasting glycemic level. These were very slight, ranging from 0 to 3 mg %, provided that the subjects adhered to a fairly uniform though unmeasured diet.

Since rather slight changes in the blood sugar were to be detected, accuracy of the analytical procedure required special attention. At the time of these studies—1936 and 1938—we used the copper-iodometric

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[1] Staub, H., *Z. klin. Med.*, 1922, **93**, 133.

[2] Kageura, N., *J. Biochem.* (Japan), 1922, **1**, 333.

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TABLE I.
Changes in Postabsorptive Blood Sugar Level of Healthy Persons Under the Influence of High Fat Diets.

Subject	Duration of high fat diet, days	Mg glucose per 100 cc venous blood		Increase, mg %
		On normal diet	After high fat diet	
C.	2	85	96	11
H.	3	87	95	8
T.*	2	72	83	11
M.	3	86	91	5
G.	3	84	89	5
S.	3	87	100	13
R.C.	3	91	100	9
H.K.	2	85	91	6
K.	1	88	94	6
E.S.	2	98	99	1

* Carbohydrate ration was decreased to 50-60 g per day.

procedure of Shaffer and Somogyi[5] with the deproteinization method later described by Somogyi.[6] The results obtained represent true (fermentable) sugar. The accuracy of the technic was within 1-2 mg %; thus any change registered in the blood sugar level, which exceeded 2 mg %, was regarded as representing an actual change.

In Table I are presented 9 cases in which high fat diet, administered for 2-3 days, caused a distinct rise in the postabsorptive blood sugar. As may be noted, the smallest increase was 6, the highest 13 mg %. In the 10th case (the last in Table I) no change had occurred in 2 days. This subject was a heavy eater, accustomed to consuming between 150 and 200 g of fat per day, with the consequence that the addition of an extra 100 g of fat for 2 days constituted a relatively less significant change in his diet and probably for this reason failed to affect his glycemic level. His postabsorptive blood sugar, as may be seen in Table I, was 98 mg % before the experiment. This was already abnormally high, most likely owing to overeating and heavy consumption of fat. (The normal variations range between 70 and 90 mg %, as shown by our statistical material (unpublished) comprising more than 1000 individuals with normal carbohydrate metabolism).

The change in the postabsorptive blood sugar is readily reversed by returning the subject from high fat to his normal diet. The 4 examples recorded in Table II illustrate the reversibility of the process. Return of the blood sugar to normal levels was effected when a high fat regime was followed by 3 days of low fat (60-70 g) diet; then again a rise took place in the blood sugar after another 2 days' course on a high fat regime. It is evident that the postabsorptive sugar level can be shifted back and forth by changing the dietary regime. Increase of the fat ration leads to an elevation, decrease of it to a lowering of the glycemic level.

TABLE II.
Reversibility of the Postabsorptive Blood Sugar Level of Healthy Persons by Changing Diets.

Subject	Mg glucose per 100 cc venous blood on		
	High fat	Low fat	High fat
R.C.	99	91	99
M.S.	98	88	100
H.H.	95	87	93
N.C.	96	85	95

In order to produce an increased blood sugar level by a high fat regime, the carbohydrate ration must not be decreased below 100 g per day; if it is decreased to about 50 g, the postabsorptive blood sugar may or may not increase. In some cases it does increase during the first or second day, but then it usually declines below the normal level. One of our subjects (Dr. T. in Table I who, with a fat ration of 200-250 g, received only 50-60 g of carbohydrate per day) showed a rise of blood sugar on the second

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[5] Shaffer, P. A., and Somogyi, M., *J. Biol. Chem.*, 1933, **100**, 695.

[6] Somogyi, M., *J. Biol. Chem.*, 1945, **160**, 69.

TABLE III.
Changes in the Postabsorptive Blood Sugar Level of Diabetic Patients Under the Influence of Low Fat Diets.

Case No.	Date	Blood sugar mg %	Decrease in blood sugar mg %	Case No.	Date	Blood sugar mg %	Decrease in blood sugar mg %
1	5-21-36	174		16	2-13-36	208	
	26	129	45		3- 4	101	107
2	6- 8	122		17	3-26	204	
	11	102	20		4- 8	103	101
3	10-19	137		18	5- 6	245	
	26	114	23		13	153	92
4	11-17	161		19	7-16	264	
	25	85	76		25	168	96
5	3-23-37	123		20	9-18	342	
	26	112	11		25	269	73
6	7-28-38	158		21	11-13	201	
	8- 1	117	41		21	108	93
7	11-22	164		22	3-27-37	222	
	28	145	19		29	173	49
8	1-10-39	110		23	7-24	195	
	17	95	15		28	150	45
9	4-26	135		24	1-27-39	371	
	5- 3	103	32		30	323	48
10	5- 4	171		25	7-19	187	
	10	126	45		25	131	56
11	5-16	147		26	8- 2	255	
	24	119	28		5	181	74
12	7- 8	125		27	10-21	231	
	10	94	31		26	120	111
13	7-11	134		28	11-27	295	
	14	116	18		12- 1	207	88
14	10-17	152		29	3- 1-40	375	
	19	127	25		4	258	117
15	11- 6	111		30	4-27-35	247	
	8	84	27		6- 1	128	119

day of the regime, but a drop to the low level of 64 mg % during the next 2 days.

These experiments were prompted by observations previously made on diabetic patients who showed a substantial decrease in the fasting blood sugar level after being placed on diets which contained 200-300 g of carbohydrate and only 50 g of fat per day. The drop occurred often in 2-3 days, in other instances it required a week or more. Only patients whose diabetic condition was not very severe were thus studied; none who had ketosis was included in the series. This was a necessary condition, since no insulin treatment could be employed during the observations. For the same reasons patients who previously had been accustomed to treatment with large doses of insulin (25 units or more per day), were excluded. Patients with coronary artery disease and passive congestion of the liver were likewise ruled out, as it is known that in this condition the blood sugar may suddenly shoot up to high levels and just as rapidly recede after the correction of cardiac decompensation.

In Table III are recorded 30 examples representative of observations comprising over 400 cases. A substantial decrease of the fasting blood sugar is evident in all instances. In general, 2 factors influence the degree of change: In the first place, the greater the interval of time between 2 sugar determinations, the greater is the drop in the hyperglycemic level. The second factor is the initial glycemic level: the higher this is before the initiation of the low fat diet, the greater will be the drop during a given period.

The effect of low fat diet follows no uniform pattern; in fact it shows great individual variations. There are cases, for instance, in which a change from high fat, low carbohydrate to low fat (50 g), liberal carbohydrate (200-300 g) diet produces an increase in the fasting blood sugar. This is, however, a transitory change, for a few days later the predominating effect of low fat pre-

TABLE IV.
Initial Rise, Followed by Drop of Postabsorptive Blood Sugar Level of Diabetics after Changing from High Fat to Low Fat Diet.

No.	Patient	Date	Blood sugar mg %
1.	E.S. ♀	April 22, 1936	325
		,, 24	335
		May 8	276
		,, 19	170
2.	P.R. ♂	June 14, 1939	108
		,, 15	124
		,, 16	107
3.	G.L. ♂	Jan. 8, 1940	187
		,, 12	232
		,, 16	190
		,, 19	171
4.	M.S. ♂	Aug. 22	151
		,, 23	168
		,, 26	140
		,, 30	108
5.	M.Sc. ♂	Dec. 8, 1942	121
		,, 9	147
		,, 11	127
		,, 12	109

vails and the blood sugar follows the usual declining course. Thus the initial rise can be misleading if one observes the changes of the blood sugar only for a few days after the change in diet. In Table IV are recorded a few cases which illustrate this phenomenon. The changes of the postabsorptive blood sugar level under the influence of diet are reversible in diabetic patients in the same way as in the normal: high fat rations, associated with restrictions of carbohydrates, augment the hyperglycemia, while low fat, liberal carbohydrate diets lead to a decrease of the blood sugar.

Summary. 1. Diets containing high fat rations and restricted amounts of carbohydrates increase the postabsorptive (fasting) blood sugar level. 2. This change takes place in healthy and diabetic individuals alike, with the difference that it is much more accentuated and obvious in the diabetic organism. Thus, the physiologic processes in the healthy and in the diabetic individual are qualitatively identical, but in the diabetic they are greatly exaggerated (Claude Bernard); in other words, the difference between the normal and the diabetic individual, in regard to the physiologic process described, is only of degree but not of kind.

15954

Effect of Rutin on Induced Proteinuria in Albino Rats.*

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Various reports have appeared in the recent literature concerning the ability of the flavone, rutin, to decrease capillary permeability. These publications are somewhat at variance with one another, and many of them deal with qualitative data. Since the permeability of the glomerular capillaries is one of the factors which influence the rate of protein excretion in the urine, and since it has been claimed that rutin changes capillary permeability, a quantitative study was undertaken to determine the effect of large doses of rutin on the rate of protein excretion following parenteral administration of bovine albumin to albino rats.

A total of 84 healthy albino rats, of both sexes, was selected at body weights of approximately 150 g each. These were separated into 4 groups, rats within each group being of the same sex. During the 72 hours of the experimental period following the initial administration of albumin all rats were kept in urine collection cages and were fed 10% dextrose in water containing 0.4% sodium chloride and vitamins of the B com-

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* The bovine albumin used in these experiments was obtained through the courtesy of Dr. J. D. Porsche from the Armour Laboratories.

The rutin used was supplied by the Abbott Laboratories.

TABLE I.
Average Autopsy Findings (per rat).

	Control rats				Rutin-treated rats			
	Group II	Group III	Group IV	Mean	Group II	Group III	Group IV	Mean
No. and sex	11 ♂	12 ♀	11 ♀	—	11 ♂	12 ♀	12 ♀	—
Body wt, g	145	146	143	145	145	147	141	144
Carcass wt, g	110	108	107	108	109	110	106	108
Kidney wt, mg	1159	1106	1129	1131	1267	1225	1147	1213
Kidney protein content, mg	191	176	172	179	196	187	178	187
Liver wt, mg	6318	7137	6845	6767	6840	7950	7222	7337
Heart wt, mg	611	597	613	607	604	619	607	610
Serum protein, g %	5.63	6.21	5.98	5.94	5.06	5.19	5.06	5.11
Serum creatinine, mg %	0.72	0.84	0.67	0.74	0.61	0.64	0.84	0.70
Creatinine excret., mg/rat/24 hr	4.73	5.22	4.99	4.98	4.62	5.34	5.04	5.00

plex. Each of the 4 groups was subdivided into equal numbers of control rats and rats to be treated with rutin. Equivalent body weights and normal proteinuria,[1] the latter having been determined one day prior to the start of the experimental period, served as the criteria. The normal proteinuria for the rats in these experiments, expressed as mg of proteinuria per rat per 24 hours, averaged 5.3 mg for the males and 3.2 mg for the females.

Sixteen cc of 6.2% bovine albumin in normal saline were administered intraperitoneally to each rat 3 times: at the start of the experimental period and 8 and 24 hours later. The controls were given subcutaneous injections of 1 cc of normal saline at the 24th, 26th, 28th, and 30th hours, and the experimental rats were injected similarly and at the same time intervals with 1 cc doses of a preparation of rutin in normal saline. The rutin concentration was 45 mg per cc, each dose being equivalent to 300 mg of rutin per kg of body weight.

Urine collections were made at 8, 24, 26, 28, 30, 32, 48, 56, and 72 hours. The urinary output from each rat was first measured and charted. Then the specimens were combined to make one urine pool for each collection period for the control animals and one for those rats receiving rutin. The animals were killed on the 72nd hour and various autopsy measurements were made. As Group I, consisting of 12 female rats, was intended to be an exploratory group, no autopsy data were obtained.

Findings are presented in Table I as average figures for the rats within each group. Gravimetric determinations of kidney protein content were made on pooled kidneys.[2] Serum protein and creatinine were measured from pooled blood specimens, the creatinine being determined by the method of Addis *et al.*[3] The protein content of the urine and serum pools was measured by a modification of the biuret method of Kingsley.[4] Addition of rutin in high concentration to specimens of known protein content did not effect the protein determinations. All biochemical measurements were made in duplicate.

Fig. 1 shows that rutin was ineffective in altering the proteinuria induced by the administration of bovine albumin. Differences in proteinuria between control animals and rats treated with rutin were no greater than those between separate control groups. We have no reason to believe that average autopsy values (Table I) revealed significant dissimilarity between control animals and those treated with rutin in regard to body, carcass, or organ weights, kidney protein content, or serum protein values. The concentration of creatinine in the serum and the rate of excretion of creatinine in the urine did not appear to be changed by the administration of rutin. No evidence of toxicity was observed,

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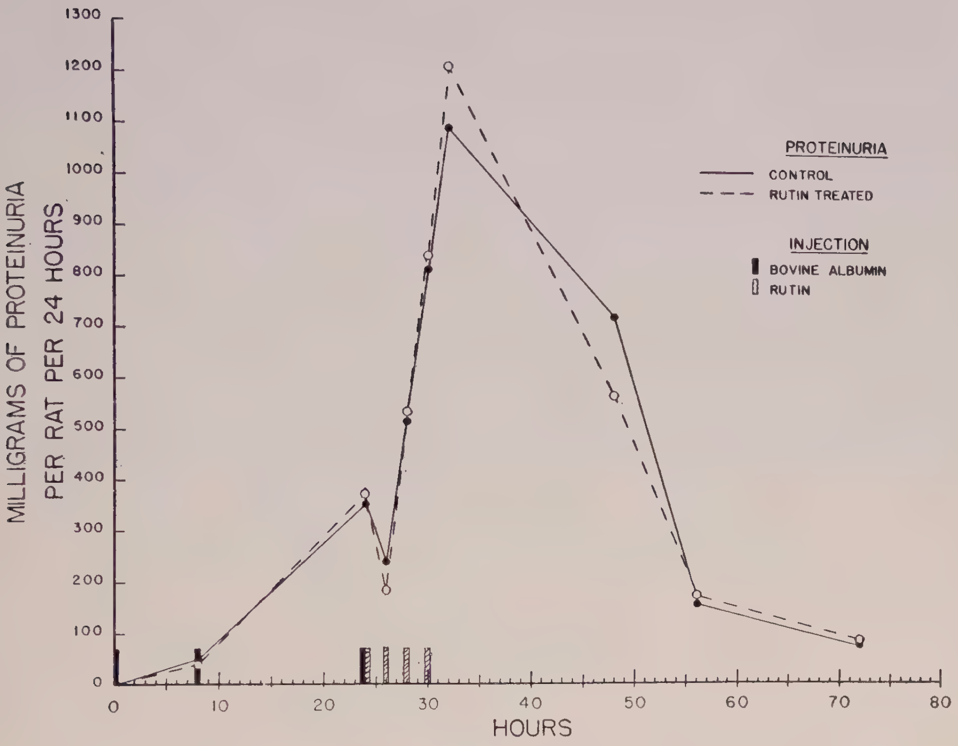

Fig. 1.
Average values for Groups I-IV, expressed as mg of proteinuria per rat per 24 hours.

despite the high dosage of rutin employed.

The urines of the rats given rutin became tinged with the yellow color of rutin in an alkaline medium one hour after the first injection of rutin, and this coloration persisted through the last collection of urine 48 hours later. When such urines were acidified, the yellow color disappeared. No such color change was noted in the blood sera, even when made strongly alkaline. These observations are similar to those described by Wawra and Webb[5] for another related flavone, hesperidin.

Summary. In albino rats with proteinuria induced by the administration of bovine albumin, rutin in high dosage did not appear to alter significantly (1) the degree of proteinuria, (2) the organ or body weights, (3) the total serum protein content, (4) kidney protein content, (5) the serum creatinine concentration, or (6) the rate of excretion of creatinine in the urine. No toxic manifestations attributable to rutin were observed. A color change in rutin solution, from yellow in alkaline medium to colorless in acid medium was noted.

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15955

Experimental Infection of Flies with Human Poliomyelitis Virus.*

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In previous work from 3 laboratories[1-5] nonbiting flies collected during poliomyelitis epidemics have been found to harbour the virus of poliomyelitis regardless of whether they have been collected at rural, suburban, or urban areas from widely scattered parts of the country. Thus, they have been found positive for virus when collected in 1941 in rural Connecticut and Alabama,[1,2] in semi-rural New Brunswick, Canada,[2] and also in Atlanta, Ga.,[3] and Cleveland, O.[3,4] In 1943, positive tests were obtained from flies trapped in January at San Antonio, Texas[2] and in August at Chicago, Ill.[5] In 1944 virus was isolated from flies collected in rural North Carolina[5] and in 1945, from those trapped in Rockford, Ill.[6]

It would seem that the rôle played by flies in the transmission of this disease has not been determined, and the fact that flies may be "contaminated" with virus in nature, does not tell whether they may act as true hosts for the virus. It is important nevertheless to answer the question of the survival of the virus in the fly especially as it pertains to possible multiplication in this insect.

The first experiments in this connection were carried out by Howard, Clark, and Flexner[7,8] who were able to recover a strain of virus from *Musca domestica* up to 48 hours after being fed on infected monkey cords. This work was confirmed 3 decades later by Bang and Glaser,[9] and by Rendtorff and Francis;[10] both groups of investigators also used *Musca domestica,* which were infected with the Lansing murine-adapted strain. Attempts to recover the Lansing strain from 4 other species of flies were unsuccessful. Bang and Glaser, however, were able to show that the GD VII strain of spontaneous mouse encephalomyelitis virus persisted in house flies in one test for 12 days. The significance of this as far as the human disease is concerned is difficult to evaluate because of the failure to establish any relationship of this virus to human or simian- and murine-adapted strains of poliomyelitis virus.

In selecting the fly species to be used in our experiments, we were influenced by the fact that blow flies *(Phormia regina)* and green bottle flies *(Phaenicia sericata)* have appeared in all the positive batches of flies from epidemic areas tested in our laboratory. Feeding habits of these flies are such that they are attracted to feces as well as common foods.[11,12] Furthermore, in a series of tests for virus carried out on the 4 most prevalent fly species collected at an epidemic in Rockford, Ill., in 1945, only *Phormia regina* were positive, whereas *Phaenicia*

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* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

† Dr. L. R. Penner assisted in these experiments while on temporary leave from the Department of Zoology and Entomology, University of Connecticut, Storrs, Conn.

[1] Paul, J. R., Trask, J. D., Bishop, M. B., Melnick, J. L., and Casey, A. E., *Science,* 1941, **94**, 395.

[2] Trask, J. D., Paul, J. R., and Melnick, J. L., *J. Exp. Med.,* 1943, **77**, 531.

[3] Sabin, A. B., and Ward, R., *Science,* 1941, **94**, 590; 1942, **95**, 300.

[4] Toomey, J. A., Takacs, W. S., and Tisher, L. A., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 637.

[5] Melnick, J. L., and Ward, R., *J. Infect. Dis.,* 1945, **77**, 249.

[6] Melnick, J. L., unpublished results.

[7] Flexner, S., and Clark, P. F., *J. A. M. A.,* 1911, **56**, 1717.

[8] Howard, C. W., and Clark, P. F., *J. Exp. Med.,* 1912, **16**, 850.

[9] Bang, F. B., and Glaser, R. W., *Am. J. Hygiene,* 1943, **37**, 320.

[10] Rendtorff, R. D., and Francis, T., *J. Infect. Dis.,* 1943, **73**, 198.

[11] Power, M. E., Melnick, J. L., and Bishop, M. B., *Yale J. Biol. Med.,* 1943, **15**, 1943.

[12] Penner, L. R., unpublished observations.

sericata, Musca domestica, Ophyra leucostoma gave negative tests.[6]

In addition, the investigations referred to above[7-10] have not, perhaps, been carried out with strains of virus or a form of virus with which flies may come in contact in nature. The strains employed have been either monkey or murine-adapted in the form of central nervous system tissue from infected animals, and in one instance Theiler's virulent strain of mouse encephalomyelitis virus.

Experimental. In the present experiments, an attempt has been made to approach, somewhat, conditions in nature. Thus, studies were designed to "infect" *Phormia regina* by allowing them to feed on virus as naturally present in stools of poliomyelitic patients. As control experiments, this species of fly as well as *Phaenicia sericata* and *Sarcophaga bullata* were infected with Y-SK and Lansing murine-adapted strains of poliomyelitis virus, and with the TO strain of Theiler's spontaneous encephalomyelitis virus of mice as it naturally occurs in the intestinal contents of laboratory mice.

All flies used in the experiments were laboratory bred. To facilitate handling, individual flies were mounted permanently by their thoraces and wings to low melting point paraffin blocks fastened on glass rods (Fig. 1) and kept in specially devised racks that could be transported easily for desirable temperature regulation. These methods are to be discussed in another paper.

Using a specially devised potometer it has been found that under the conditions of these experiments *Phaenicia sericata* average approximately 0.005 cc of fluid although some individuals may take up to 3 times as much (0.015 cc). *Phormia regina* average about twice as much food per meal (0.01 cc) as does *Phaenicia sericata* and some specimens take up to 0.04 cc. *Sarcophaga bullata* may take considerably more than this but it is so temperamental in feeding that accurate measurements are rather difficult to make.

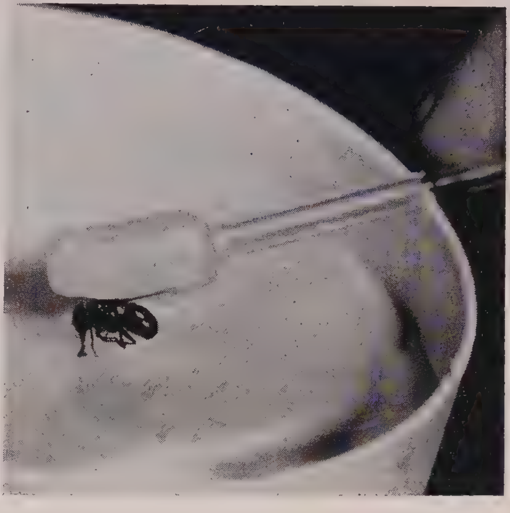

Fig. 1.
Phormia regina permanently mounted on a paraffin block. The fly is shown feeding on a solution of sucrose.

Results. When murine-adapted strains of poliomyelitis virus or the intestinal TO strain of Theiler's virus were fed to flies (*Phaenicia sericata, Phormia regina,* and *Sarcophaga bullata*) virus could often be recovered within 72 hours from the bodies and excreta of the insects. It appeared that virus could be isolated from the abdomens more readily than from the heads and thoraces. Occasional tests from any part of the fly were positive from the 3rd through the 5th days, and none yielded virus thereafter. When a biologically inert substance, such as carmine, was fed, it was found to be excreted in gradually decreasing quantities for 6 days following feeding. Only traces remained in the excreta of the 6th day.

These data are presented in Fig. 2 in which the results of 11 experiments on murine viruses are shown. In most of these experiments the flies were kept at night at 10°; every other day they were brought out and held at room temperature (about 25°) for 6 to 8 hours. During their stay at the higher temperature they were fed on molar sucrose solution to which at times autoclaved stool extracts were added to provide additional nutrients. A few experiments were carried out in which the flies were held at a constant temperature of 27°. Such flies were fed daily. Of these 11 experiments, *Phaenicia sericata* were used in 7 (3 with Lansing strain, 1 with Y-SK, and 3 with TO); *Phormia regina* were

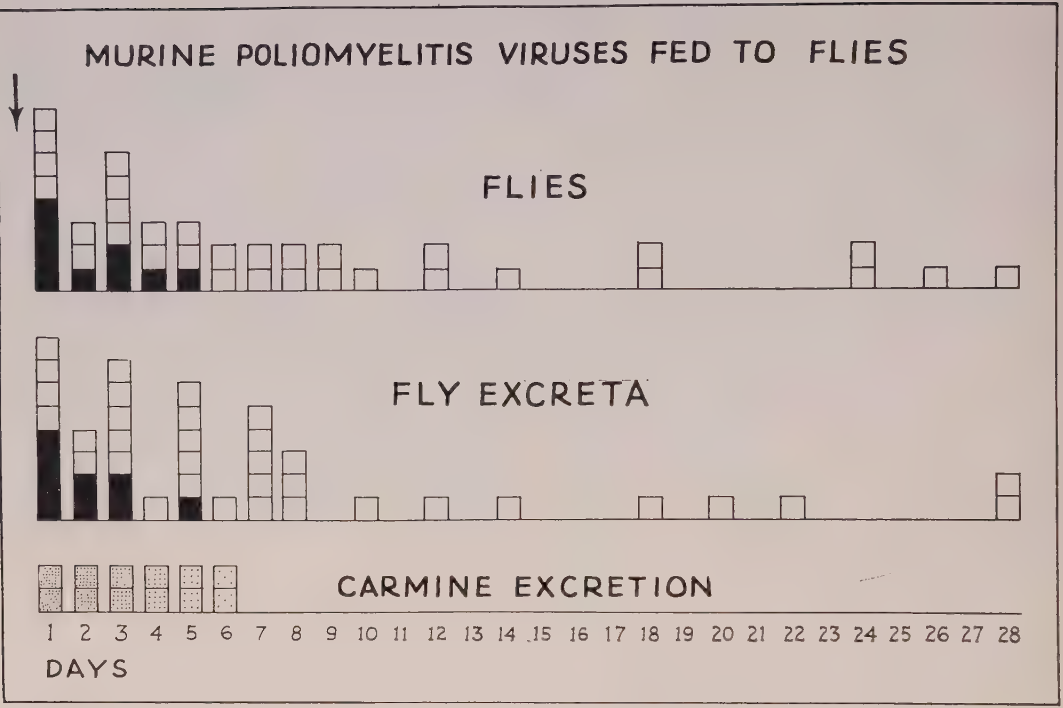

Fig. 2.

Combined results of 11 experiments in which murine poliomyelitis viruses (Lansing, Y-SK and TO) have been fed to *Phormia regina*, *Phaenicia sericacta*, and *Sarcophaga bullata*. White squares indicate negative tests for virus; black squares, positive tests. The relative amount of carmine in the excreta is indicated by the degree of stippling.

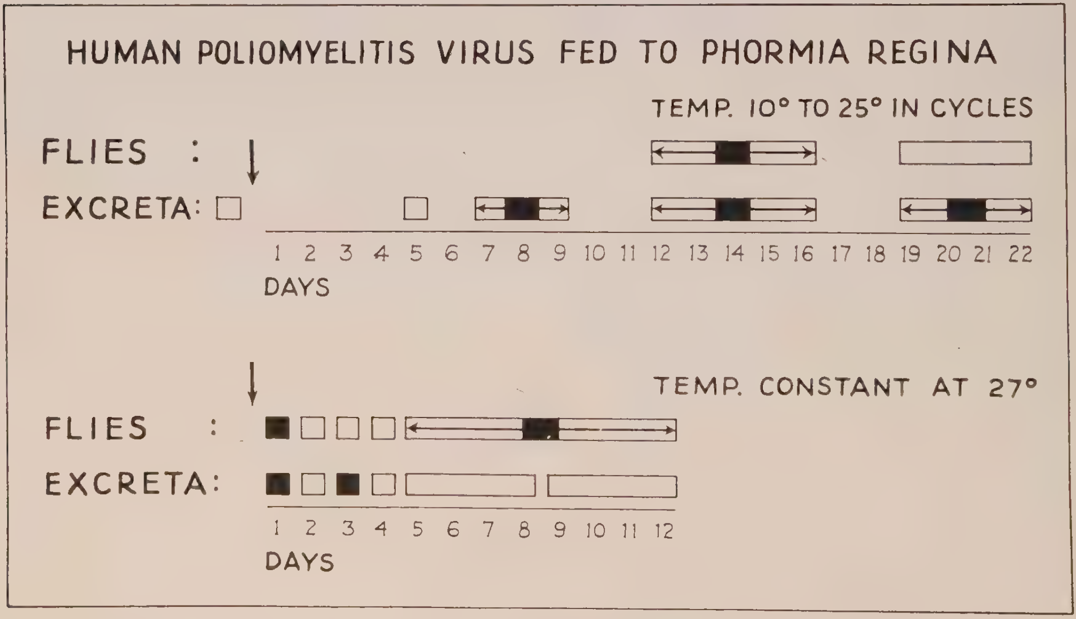

Fig. 3.

Poliomyelitis virus in flies and in their excreta after being fed on stools obtained from patients in the acute stage of the disease. White squares and rectangles indicate negative tests for virus; black areas indicate positive tests. When a pooled sample representing more than one day's collection was used and found positive, the arrows indicate the days covered in the pool.

used in 2 (1 with Y-SK and 1 with TO); and *Sarcophaga bullata* with 2 (both with TO). In 6 of these experiments the flies were fed on virus only once; in 3, they were fed virus for 3 consecutive days, and in 2, for 6 consecutive days. The last day of virus feeding was regarded as the "zero" day. No differences were detected using different species of flies or different strains of virus.

The results were markedly different with human poliomyelitis virus (NYC-44 strain) fed to *Phormia regina,* as shown in Fig. 3. Two experiments were carried out: In the first, 20 *Phormia* were fed once on a virus preparation from human stools from poliomyelitic patients. After the virus feeding they were treated as above with cyclic temperature changes from 10° to 25°. Due to mechanical difficulties the cooler temperature was not always constant, but it was not below 9° nor above 13° except at one period (18th to 22nd days after feeding) when it ranged between 13° and 20°.

Of the 20 flies, 1 died on the 12th and 1 on the 16th day. These 2 flies were pooled with 5 which were removed from the racks on the 14th day; this sample proved positive in a Siamese monkey *(Macaca irus valida)*; tests in a rhesus *(M. mulatta)* and 2 Philippine cynomolgus monkeys *(M. cynomolgus)* were negative. Of the remaining 13 flies 1 died on the 19th day, 2 died on the 21st day, 1 died on the 22nd day, and 9 were sacrificed on the 22nd day. These were pooled, and yielded negative tests in 4 monkeys of similar species distribution as those used on the first test.

Excreta collected before the virus feeding and on the 5th day following the feeding were negative. However, samples obtained on the 7th-9th days, 12th-16th days, and 19th-22nd days, all gave positive tests for virus. Only 13 flies contributed to the excreta after the 14th day.

The second experiment was carried out with 200 *Phormia.* These were kept at a constant temperature of 27°. They were fed once on virus and then daily on the sterile nutrient solution. On the first day following the feeding, their excreta were collected and found positive for virus. The excreta collected on the 4th, 5th-8th, and 9th-12th days were negative.

When the flies themselves were tested, a positive test was obtained with a sample of 50 flies sacrificed within 24 hours after feeding on virus. Samples of 16 to 50 flies taken from the 2nd day through the 12th day were negative when tested in rhesus, green, or Siamese monkeys. A third of the sample collected on the 4th day and a third of the sample collected on the 5th-12th days were each tested in a cynomolgus monkey. The monkey receiving the sample from the latter period developed typical experimental poliomyelitis.

Discussion. These experiments illustrate a difference which obtains when human poliomyelitis virus rather than simian- or murine-adapted strains, is used in attempting to evaluate the fly as a potential host of the virus in nature. Whereas murine-adapted viruses or Theiler's TO strain behaved as inert material in the fly, being eliminated in decreasing quantities over a 5-day period, human intestinal virus was found in the fly at the end of 3 weeks.

The difference between the murine and the human strains cannot be explained on the basis of titer of infective material. Thus the Lansing strain preparation, which was fed as a 10% emulsion of infected CNS, could be diluted a further 500 times and still be found infective for mice in 0.03 cc amounts inoculated intracerebrally. In monkeys, furthermore, this strain[13] as well as the Y-SK[6] has a higher titer than in mice (about 100-fold) which may be related to the larger inoculum (1.0 cc) given to monkeys.

Too few test monkeys were used to place significance on the negative tests which were followed by positive ones. This occurred in the first experiment (Fig. 3) when a negative test on the 5th day excreta was followed by positive ones on the excreta of the next 2 weeks. Likewise in the second experiment (Fig. 3) the negative tests in flies from the 2nd through the 4th days were followed by a positive one on a later sample.

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[13] Bodian, D., and Cumberland, M. C., *Am. J. Hygiene*, 1947, **45**, 226.

The result, if any, of the cyclic temperature variation in prolonging the persistence of virus in the fly cannot be evaluated on the basis of the available data. The only thing that can be stated is that under the same conditions of alteration in temperature used with the human strain, murine-adapted strains could not be found beyond the 5th day.

Quantitative virus balance studies were not attempted in these experiments. Without knowledge of the exact amounts of virus taken up by the flies, of the amounts excreted in the first days after the feeding, and the amounts excreted in the 2nd and 3rd weeks following the feeding, it is not possible to answer the important question of whether virus multiplies in the fly. These results however unlike those with the murine-adapted strains, are compatible with the possibility of multiplication.

Summary. Human poliomyelitis virus, as naturally present in stools of poliomyelitic patients has been fed to blow flies, *Phormia regina*. After this feeding, virus was found in the flies for 2 weeks, and in their excreta for 3 weeks.

Results of this type were not encountered when murine-adapted strains of poliomyelitis virus and Theiler's TO strain of spontaneous encephalomyelitis of mice were used. These strains behaved like biologically inert material, such as carmine, in *Phormia regina, Phaenicia sericata* and *Sarcophaga bullata*; following their ingestion they were found in gradually decreasing quantities for a period of 5 days.

15956

Direct Isolation of Mumps Virus in Chick Embryos.*

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Mumps virus has been adapted to embryonated hen's eggs by the inoculation of relatively large doses of infected monkey parotid by Habel[1] and Levens and Enders[2]. Beveridge, Lind and Anderson[3] using penicillin and sulfonamides as bacteriostatic agents, were able to isolate 4 strains of mumps virus from 12 specimens by direct inoculation of human saliva into the yolk sac of 5 or 6 day embryos. These investigators also isolated one strain by the inoculation of the amniotic sac of 9 or 10 day old embryos. While virus was isolated from 5 of 13 specimens by either yolk sac or amniotic sac inoculation, bacterial contamination was a frequent source of difficulty. Several specimens could not be passed satisfactorily for this reason.

The present report deals with the successful isolation of mumps virus from 8 of 9 patients by the inoculation of saliva into the amniotic sac of 7 or 8 day old chick embryos.

Materials and Methods. Saliva was collected from patients ill with mumps during the acute, inflammatory stage of the disease. This material was emulsified with approximately equal parts of sterile infusion broth. Centrifugation at about 3000 r.p.m. in an angle centrifuge for 15 minutes served to deposit large particles, and presumably, a portion of the bacteria. Penicillin and streptomycin were added to the supernatant to a final concentration of 250 and 2500 units per milliliter, respectively, and the mixture of

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* Supported in part by a grant from the Research Grants Division, National Institute of Health, United States Public Health Service.

[1] Habel, Karl, *Public Health Rep.*, 1945, **60**, 201.

[2] Levens, J., and Enders, J., *Science*, 1945, **102**, 117.

[3] Beveridge, W. I. B., Lind, P. E., and Anderson, S. G., *Australian J. Exp. Biol. and M. Sc.*, 1946, **24**, 15.

TABLE I.
Isolation of Mumps Virus Directly from Patients by Amniotic Sac Inoculation of Chick Embryos.

Patient	Age	Day of disease	Virus isolation	First positive embryo passage	Complement fixation test on patient's sera: Acute Day	Acute Titer	Conv. Day	Conv. Titer
P.L.	4 yr	3	+	1st	—	—	—	—
S.L.	18 mo	1	+	”	—	—	—	—
A.L.	5½ yr	4	+	2nd	—	—	—	—
E.J.	26 ”	2	+	1st	2	<1/4	15	1/256
		5	0	(3 neg.)				
M.W.	8 ”	2	0	(3 ”)	2	1/64	23	1/64
T.C.	23 ”	5	+	2nd	5	<1/8	—	—
E.L.	26 ”	1	+	1st	1	<1/8	16	1/64
J.G.	45 ”	1	+	”	1	1/8	14	1/8
M.A.	33 ”	6	+	”	6	1/16	12	1/256

saliva and antibiotics inoculated without further treatment.

White Leghorn eggs, incubated 8 days at about 38°C., with the air sac uppermost, were routinely used for inoculation. After preliminary candling, a circle 12-15 millimeters in diameter was inscribed over the air sac with a dental drill. This circle of shell was removed with a sterile scalpel. A large drop of sterile broth was then deposited on the shell membrane, and the point of a forceps gently thrust through the drop of fluid and through the shell membrane but not through the underlying chorio-allantoic membrane. The shell membrane was gently removed with forceps. The amniotic sac could then be inoculated under direct vision. One-tenth ml was inoculated into each embryo, and the specimens were incubated at 35-36°C. for 7 days. Usually 10 eggs were inoculated with each specimen.

At the end of incubation, the shell and chorio-allantoic membranes were cut away, and the amniotic fluid removed with a capillary pipette. The fluid was tested for the presence of virus by mixing 0.5 ml with an equal volume of 0.5% pooled, washed chicken erythrocytes, and observing the presence or absence of the characteristic "pattern" of agglutinated cells.[2]

Fluids which gave positive hemagglutination reactions were repassed amniotically. Amniotic membranes of specimens giving negative hemagglutination tests were ground with sterile powdered pyrex glass, mixed with the homologous amniotic fluids, centrifuged briefly, and re-inoculated. Three passages were made before a specimen of saliva was considered negative for mumps virus.

Identification of the infective agent as mumps virus was performed by means of the complement fixation reaction, using acute and convalescent human mumps serum and infected amniotic fluid as antigen.[1] In addition, two specimens were each inoculated in 1 ml amounts into the parotid ducts of two *Macacus rhesus* monkeys. The one monkey of each pair showing the greatest parotid swelling was sacrificed at the period of maximum swelling. The virus was re-isolated in each case from emulsified parotid tissue by amniotic inoculation. The other monkey of each pair was allowed to live for several weeks, and in each instance demonstrated clinical and serological evidence of mumps.

Results. Specimens of saliva from 9 patients with a clinical diagnosis of mumps have been examined. Mumps virus has been isolated from 8 of these patients. In instances in which blood was available for examination, complement fixation tests were performed, using allantoic fluid containing a laboratory strain of mumps virus as antigen.† Data regarding these cases are presented in Table I.

One of these patients, M. W., had typical bilateral mumps 4 years before the illness recently investigated. The more recent illness was mild, characterized by slight fever, malaise, and unilateral parotitis. Attempted virus isolation gave negative results. Complement fixation tests, although yielding higher values than expected, showed no rise during convalescence, and thus failed to confirm the clinical diagnosis of mumps made by her father, a physician.

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† This strain of virus was kindly supplied by Dr. John Enders, Boston, Mass.

The technique described should prove useful in many phases of clinical investigation of mumps, especially for the detection of virus in bodyfluids and secretions at different stages of the disease and in the presence of various complications. Such studies are now in progress.

Summary. A technique for the direct isolation of mumps virus by inoculation of saliva into the amniotic sac of chick embryos is presented in detail. Employing this technique, mumps virus was isolated from 8 of 9 patients with clinical mumps.

15957

Electroencephalogram of Cats Subjected to Repeated Minimal Convulsive Doses of Electricity.

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In a previous study it was shown that the cat could survive a surprisingly large number of repeated convulsions when these were induced by minimal convulsive doses (MCD) of electricity.[1] In the course of such studies it became of interest to determine what electroencephalographic (EEG) alterations took place, especially since the gross behavioral changes of the animals were not particularly striking. A number of other investigators have already described the EEG in several species of normal animals—*e. g.* cat, dog, rabbit and monkey. Reports concerning the brain wave activity following electrically-induced convulsions, however, are relatively meager[2-6] and no data has so far been made available depicting the EEG changes in animals as their convulsions were continued.

It is, therefore, the purpose of this communication to report such a study in which the progressive changes in the electroencephalogram of cats subjected to frequently repeated convulsions were observed.

For this purpose 12 adult cats were subjected to repeated electrically-induced convulsions at approximately 5 minute intervals with minimal convulsive doses of electricity (alternating current). Solder electrodes were applied 20 mm cephalad to the external occipital protuberance and 10 mm from the midline on each side of the head which corresponded to bilaterally homologous areas of the underlying occipital lobes. These electrodes were held in contact with the shaved scalp previously treated with electrode paste (Sanborn) by the aid of parlodion and were led into the Garceau electroencephalograph. All animals were kept in a shielded box during recordings.

Results. Of the 12 cats employed, 7 succumbed and 5 survived. Because the electroencephalographic patterns were essentially similar in all animals the results obtained can best be represented by the following three plates.

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[1] Rubinstein, H. ~~S., and~~ Kurland, A. A., in press.

[2] Lowenbach, H., and Lyman, R. S., *J. Neurol. and Psychiat.*, 1940, **3**, 336.

[3] Bawera, S. E., Lewis, N. D. C., Pacella, B. L., and Kalinowsky, L., *Tr. Am. Neuro. Assn.*, 1942, **68**, 31.

[4] Rheinberger, M. B., and Jasper, H. H., *Am. J. Physiol.*, 1937, **119**, 186.

[5] Clark, S. L., Chambers, W., and Baker, C. F., *Anat. Rec.*, 1942, **82**, 482.

[6] Clark, S. L., and Ward, J. W., *J. Neurophysiol.*, 1945, **8**, 99.

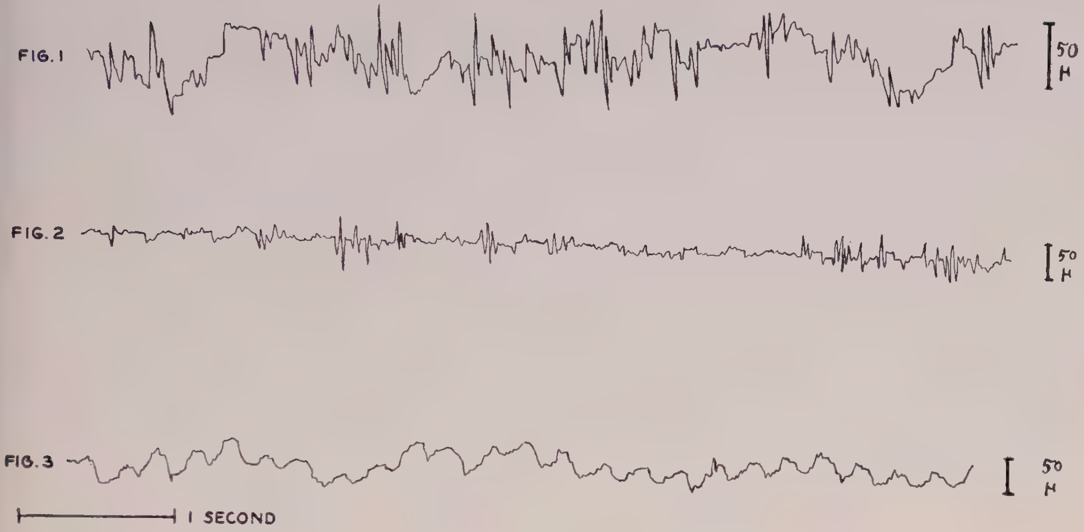

PLATE I (CAT NO. 15)

Fig. 1. Normal tracing with animal somewhat tense disclosing a 14 per second medium voltage pattern interspersed by occasional high voltage slow waves.

Fig. 2. Tracing obtained following the 24th convulsion some 2 hours and 15 minutes after the first convulsion. Only occasional bursts of 14 per second medium voltage waves are seen, the predominating pattern consisting of 9 per second medium and low voltage waves.

Fig. 3. Tracing following the 52nd convulsion 4 hours after the first convulsion. Four per second, medium and low voltage waves are dominant. The cat died 2 hours later following its 64th convulsion.

Plate 1—discloses a typical record (of cat no. 15; wt. 5¼ lbs.) through 4 hours of electro-shock convulsions.

Plate 2—discloses a more detailed electroencephalogram taken at 1 hour intervals on a cat (4¼ lbs) which finally succumbed to repeated electro-shock convulsions.

Plate 3—is a record taken from a cat (no. 23) weighing 5 lb. which survived 95 convulsions over a period of 8 hours.

Discussion. It will be observed that 2 distinct brain wave patterns may be considered normal for the adult cat. The one is the 8 to 12 per second medium voltage rhythm in the relaxed cat; the other is the more rapid (usually 14 to 21 per second) medium voltage rhythm in the tense animal (plate 2, Fig. 1). Since the degree of tenseness and alertness usually go together one may infer that the more rapid pattern is indicative of increased alertness.

Animals subjected to repeated electrically-induced convulsions disclose a rather characteristic change in the electroencephalogram. First, the *frequency gradually decreases and the voltage gradually increases.* This continues until continuous slow *high voltage waves* replace the normal pattern. As the experiment continues the slow waves persist but the voltage begins to diminish. As the experiment is pushed the low voltage becomes fixed but a *temporary rise in frequency* (to a normal 9 per sec.) may be seen. Finally, the voltage approaches zero and the *waves become ill-defined* as death approaches.

This seems to indicate that repeated convulsions lead to a gradual depression of cerebral cortical irritability only to be interrupted by a short-lived rise in excitability shortly before death ensues. This presumed increase in cerebral cortical "apathy" seems to be substantiated by the observation that the convulsive threshold rises as the experiment progresses.[1] This study also shows the surprising degree of recovery in the cat in the brief space of 3 weeks after as many as 95

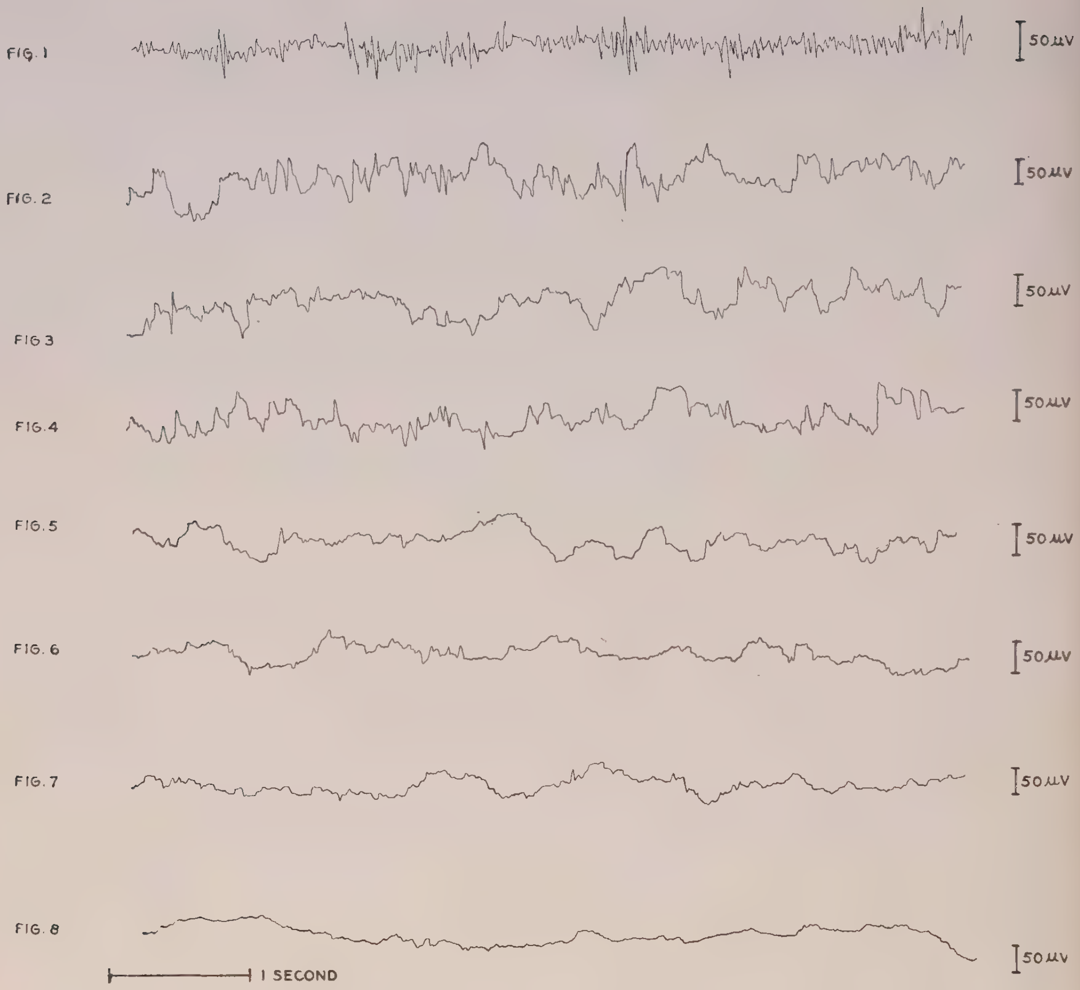

PLATE 2 (CAT NO. 16)

Fig. 1. Control tracing; animal is tense; 21 per second *medium voltage* rhythm.

Fig. 2. Tracing obtained after 10th convulsion (1 hour after beginning of experiment). Nine per second medium voltage rhythm interspersed with fairly frequent slow (2-4/sec.) *high voltage waves.*

Fig. 3. Following 18th convulsion (2 hr after onset) 8-10 per second medium and low voltage rhythm interspersed with more frequent slow high voltage waves.

Fig. 4. Following 28th convulsion (3 hr after onset) a 7 per second high voltage rhythm interspersed with short runs of 9 per second low voltage waves.

Fig. 5. Following the 36th convulsion (4 hr after onset) continuous slow wave activity of low, medium and high voltage.

Fig. 6. Following the 42nd convulsion (5 hr after onset) continuous low voltage waves varying from 4 to 8 per second.

Fig. 7. Following the 60th convulsion (7 hr after onset) continuous low voltage of 8-9 per second frequency interspersed by an occasional slow medium voltage wave.

Fig. 8. Following the 70th convulsion (8 hr after onset). Shifting base line with extremely low voltage with occasional ill-defined 2-4 per second waves. The animal died 25 minutes later.

convulsions. Recovery has also been observed in the convulsion-induced abnormalities of the human EEG. The human receiving electro-shock therapy, however, is never subjected to the concentration of treatment to which these experimental animals were ex-

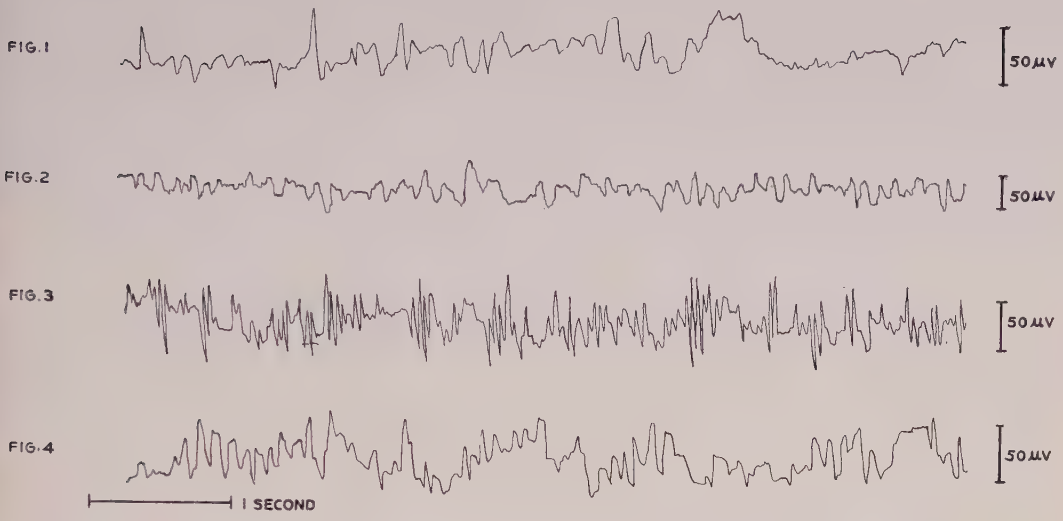

PLATE 3 (CAT NO. 23)

Fig. 1. Tracing obtained 7 days after the last convulsion when the cat was relaxed. The 7 per second medium voltage rhythm is interspersed by occasional higher voltage notched waves.

Fig. 2. 18 days after the last convulsion taken when cat was relaxed showing 8 per second medium voltage waves interspersed by an occasional 5-6 per second high voltage wave.

Fig. 3. Tracing obtained on the same day of tracing immediately above when the cat was tense. Thirteen per second medium voltage waves interspersed by an occasional slow high voltage wave.

Fig. 4. 21 days after the last convulsion taken when cat was relaxed showing an 8-10 per second medium voltage rhythm interspersed by an occasional slow high voltage wave.

posed. Even so, the disappearance of abnormalities in the human is a much slower process so that months may elapse before normal records are obtained. Kalinowsky and Hoch[7] reviewing this subject find that in the human as few as 6 convulsions (usually administered over as many or more days) will lead to slow high voltage waves which may require 1 to 5 weeks for recovery. In patients receiving 7 to 12 convulsions the recovery process may extend from 1 to 3 months. When more than 12 treatments are given recovery may require 2 to 6 months.

More rapid recovery occurs in the monkey[3] but in these animals, too, the treatment was given in relatively non-concentrated dosage. Considering the intensity of the treatment administered to the cat (in these experiments) the rapidity of recovery (21 days after 95 convulsions) is truly amazing. This was best exemplified by the gross behavior of the surviving cats. In such animals hopping and placing reactions, gait, feeding habits, preening and general alertness recovered to a degree which was indistinguishable from the untreated control cat.

Summary and Conclusion. Electroencephalographic studies were carried out in a series of 12 adult cats subjected to minimal convulsive doses of electricity repeated at approximately 5 minute intervals. It was observed that the 8 to 12 per second medium voltage rhythm of the normal relaxed cat and the 14 to 21 per second medium voltage rhythm of the normal tense cat were gradually and successively replaced by decreased frequency, lowering of voltage, continuous slow high voltage waves, fixed low voltage, temporary 9 per second (but still low voltage) waves and decreasing voltage to zero as death approaches. In the cat surviving as many as 95 convulsions the recovery of the altered electroencephalogram occurred 21 days after the last convulsion.

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[7] Kalinowsky, L. B., and Hoch, P. H., Shock Treatments and other Somatic Procedures in Psychiatry, Grune and Stratton, N. Y., 1946.

15958

A Method Applicable to the Standardization of Influenza Virus Vaccines.*

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One of the problems that came to the fore as a result of the successful trials of influenza vaccination was that of establishing standards for the control of vaccine potency. In the investigations of the Commission on Influenza[1] the vaccine employed in studies in man had been tested for capacity to immunize mice against intranasal infection. The results of such tests[2] were then used to define a standard of potency.

Because of certain features of the mouse immunization test,[3] it seemed desirable to investigate other methods that might be used for estimating potency in order to find a more sensitive and simpler procedure that required less time for completion. For this purpose the hemagglutinating property[4] of the virus provided an obvious lead. However, when an attempt was made to correlate hemagglutinating and immunizing capacities, certain difficulties were encountered. One of the chief difficulties was the matter of the relatively greater sensitivity of the methods for measuring the hemagglutinating factor as compared with the methods used for measuring immunizing effect. At best, it has been possible thus far merely to ascertain the existence of a rough correlation between these two properties.[2,5–7]

In the course of investigating this question further, a technique was employed for measuring, quantitatively, the antigenic content of preparations of influenza virus. For convenience the test is referred to as the antigenic extinction titration. It is desired to report the details of the method at this time because of its possible application to the standardization of influenza virus vaccines. The test to be described is not suggested as a direct test of the immunizing capacity of a vaccine, but rather as a quantitative test for the amount of antigen that is capable of inducing antibody formation in the mouse. Although the results may reflect immunizing effect, the present report does not deal with this question. The problem of the relationship between hemagglutinating activity, the capacity to induce neutralizing antibody, and immunity will be the subject of a later communication.

Procedure. The purpose of the test is to determine how far an antigen, or vaccine, may be diluted and still induce a measurable antibody response in groups of mice given single intraperitoneal inoculations. The procedure may best be described by illustrating its application to the determination of the antigenic extinction titer of three different preparations

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[*] This investigation was conducted under the auspices of the Commission on Influenza, Army Epidemiology Board, Office of The Surgeon General, U. S. Army, Washington, D. C.

[†] Rockefeller Foundation Fellow 1946-47.

[1] a. Members of the Commission in Influenza, Army Epidemiological Board, *J. A. M. A.*, 1944, **124**, 982; b. Francis, T., Jr., *Am. J. Hyg.*, 1945, **42**, 1; c. Rickard, E. R., Thigpen, M., and Crowley, J. H., *Am. J. Hyg.*, 1945, **42**, 12; d. Hale, W. H., and McKee, A. P., *Am. J. Hyg.*, 1945, **42**, 21; e. Eaton, M. D., and Meiklejohn, G., *Am. J. Hyg.*, 1945, **42**, 28; f. Hirst, G. K., Plummer, N., and Friedewald, W. F., *Am. J. Hyg.*, 1945, **42**, 45; g. Salk, J. E., Menke, W. J., Jr., and Francis, T., Jr., *Am. J. Hyg.*, 1945, **42**, 57; h. Magill, T. P., Plummer, N., Smillie, W. G., and Sugg, J. Y., *Am. J. Hyg.*, 1945, **42**, 94.

[2] Francis, T., Jr., *Am. J. Hyg.*, 1945, **42**, 1.

[3] Francis, T., Jr., *J. Exp. Med.*, 1939, **69**, 283.

[4] Hirst, G. K., *Science*, 1941, **94**, 22; *J. Exp. Med.*, 1942, **75**, 49.

[5] Pearson, H. E., *J. Bact.*, 1944, **48**, 369; Stanley, W. M., *J. Exp. Med.*, 1945, **81**, 193.

[6] Henle, W., and Henle, G., *J. Exp. Med.*, 1947, **85**, 347.

[7] Studies to be published.

of formalinized allantoic fluid containing the PR8 strain of influenza virus Type A. The 3 preparations had been heated for different periods in studies of the rate of thermal inactivation of the antigenic factor.[7]

The test materials were diluted serially, in 2-fold steps, using beef heart infusion broth. The range of dilutions tested is shown in Table I. A single inoculation of 0.5 cc of the respective dilutions was given, intraperitoneally, to each of 10 mice, the weights of which were approximately 18 to 22 g.

Two weeks after inoculation all mice were bled. Bleeding was done by amputating the right fore-leg as close to the attachment to the trunk as possible. This method for collecting blood is far simpler than bleeding from the heart. The yield of serum from 10 mice of the size used here is 2 to 3 cc. For the bleeding, the mouse is lightly anesthetized with ether and is then held in the left hand so that the right fore-leg of the animal is the most dependent part. The mouse is kept in anesthesia by holding its nose against the lower end of a slanted tube that is open at both ends and contains a plug of cotton saturated with ether. When the foreleg is amputated close to the trunk, the skin retracts and the spurting blood is collected in a test tube. If the animal survives it is then sacrificed with chloroform. It has not been found necessary to use an antiseptic on the skin in order to obtain a sterile sample since the serum is heated at 56° C. for one-half hour after prompt separation from the clot and the red cells.

Finally, for the detection of neutralizing antibody in the pooled serum samples, the procedure followed was essentially the same as the one described by Hirst,[8] employing the chick embryo as the indicator for virus neutralization. Serum and virus were mixed in equal volumes and incubated for 30 minutes at 35° to 37° C. before inoculation into 10 to 11 day old embryonated eggs. Four eggs were used for each serum-virus mixture and 0.1 cc was introduced intra-allantoically. The serum was used undiluted, and the virus, in the form of infectious allantoic fluid containing the same passage of PR8 virus present in the vaccine, was diluted in beef heart infusion broth so as to contain approximately 1000 50% egg-infective doses. For control, a sample of normal serum was mixed with the virus dilution used in the test. A virus titration in eggs was also done. The diluent used for the virus titration was beef heart infusion broth. These controls were incubated in the same way as the test samples.

Following inoculation the eggs were incubated for 2 days at 35° to 37° C. At the end of this period 0.5 cc of allantoic fluid was removed from each egg to test for the growth of virus. This was done by adding to the fluid removed from each egg an equal volume of 0.5% suspension of washed chicken red blood cells. The multiplication of virus in the egg is indicated by the presence of sufficient virus hemagglutinin to cause the cells to settle in a characteristic pattern.[9]

The results of this experiment are shown in Table I. It will be noted that the pool of serum from the mice inoculated with the lower dilutions of vaccine contained sufficient antibody to neutralize the virus; whereas, no detectable neutralizing antibody was present in the serum from the mice given the highest dilutions of the respective materials that were tested. It is evident from these representative data that a point can be defined, even in a 2-fold dilution series, at which the serially diluted test material no longer contains sufficient antigenic material to induce the formation of a measurable amount of antibody.

The question of the interpretation of the end-point deserves some comment. Since the antigenic extinction titer expresses the extent to which the antigen can be diluted and still induce antibody formation in the mouse, one of 2 criteria may be adopted for estimating the end-point of the titration: The end-point may be considered to be the highest dilution of antigen that induces sufficient antibody in the pool of serum from 10 mice to neutralize completely the amount of virus used in the neutralization test (approximately 100 to 1000 50% egg-infective doses). Alternatively, the end-point might be considered as the highest dilution of antigen that induces the formation of sufficient antibody to neutralize enough of the virus used in the test so that infection occurs in not more than half of the eggs inoculated. The latter criterion is preferable, and for this reason it would be better to use 6 eggs rather than 4 eggs for each serum-virus mixture.

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[8] Hirst, G. K., *J. Immunol.*, 1942, **45**, 285.

[9] Salk, J. E., *J. Immunol.*, 1944, **49**, 87.

The results obtained using this method for the determination of the antigenic extinction titer of a vaccine containing 3 strains of virus will be presented later in this report.

A Consideration of the Variables Involved in the Test. 1. *The influence of the quantity of virus used in the neutralization test upon the titer of neutralizing antibody in the serum.* Since the virus neutralization test is employed here to detect the presence of neutralizing antibody in a fixed dilution of serum, it appeared desirable to determine the influence of varying the dose of virus upon the sensitivity of the reaction. This was done in the following way. Serial dilutions were made of the 2 samples of mouse serum, one having a low antibody titer and the other a high titer. The high titer serum was obtained from mice given one intraperitoneal inoculation of 0.5 cc of a 1:4 dilution of unformalized allantoic fluid containing the PR8 strain (F198 M593 E60); the low titer serum was obtained from mice given a 1:1024 dilution of the same material. Each serum dilution series was set up in quadruplicate and a different concentration of virus was added to each. The respective serum-virus mixtures were inoculated into eggs and after 2 days' incubation the allantoic fluids were tested for the presence of virus hemagglutinin. The results are shown in Table II.

It is apparent from these data that variation in the number of infective doses from approximately 10 to 1000 results in no more than a 2-fold difference in antibody titer. Therefore, unless the antibody content of a particular sample of serum, when used undiluted, is near the limit of detection, then the quantity of virus used in the neutralization test can be varied within rather broad limits without affecting the results of the antigenic extinction titration. As the test is set up, an all-or-none effect should occur except under circumstances where the actual antibody titer of the serum sample is in the region of 1:2. Serum samples of borderline titer are found only in the region of antigenic extinction.

2. *Stability of the antigens used in the virus neutralization test.* Throughout these studies the preparations of antigen employed in the neutralization test were allantoic fluids containing different strains of active virus. These materials were stored, without preservative, at 4°C. A systematic study has not been made of the stability of the infective properties of the various strains used, but the data shown in Table III summarize the experience gathered thus far. It is evident that the preparations are sufficiently stable in the liquid state in the ordinary refrigerator, so as to be useful without re-titration immediately before use, within limited periods of time. The significance of the differences in stability of the infective titer of the different preparations shown in Table III has not been determined.

3. *The influence of the interval between inoculation and bleeding of mice upon serum antibody titer.* In the course of these experiments it was found that the interval between inoculation and bleeding has a marked effect upon the titer of serum antibody. This finding, which is illustrated in Table IV, has been studied more extensively in a series of experiments yet to be reported. The fact that the titer of neutralizing antibodies increased progressively, in the pooled serum collected at intervals between the 7th and 21st days after inoculation, was of considerable interest. It was of further interest that the titer of antibody, as measured by the agglutination-inhibition reaction, approached its maximum level in 7 days and did not continue to increase thereafter as did the level of the antibody measured by the *in vivo* technique. That the results of the agglutination-inhibition test measured antibody and not some nonspecific factor is indicated by the inhibition of agglutination by the homologous Type A virus and the absence of any inhibition of the

agglutinating effect of the Type B virus. The implications of these observations will be discussed in a more detailed report to be published. For the present purpose it is suggested that mice be bled 14 days after inoculation.

4. *The importance of strain and line specificity.* The test for antigenic extinction titer was done on the vaccine now being used as the standard of reference by the National Institute of Health. It is referred to as the Provisional Control Vaccine (Lot F2) and represents a lot of material of average potency, prepared by adsorption-elution using the red cells of the embryo.[10–11] This control vaccine, which was made by a manufacturer of biologicals, contains the prescribed strains, *i.e.*, PR8 and Weiss, both Type A, and Lee, Type B. The experiment to be described is presented because the findings emphasize the importance, particularly as it applies to the preparation and potency testing of vaccines, of antigenic variations that occur under diverse laboratory passage of the same strain.

The experiment was carried out in essentially the same manner as that described in the experiment shown in Table I. It differed, however, in that 26 g mice were used rather than 18 to 22 g mice. The results of the present test are summarized in Table V. It will be noted that the sera were tested for antibody to the PR8 and Weiss strains of Type A influenza virus and to the Lee strain of the Type B virus. Moreover, 3 different lines of the PR8 strain were compared.

The reason for making the comparison between the 3 different lines of the PR8 strain was as follows. In the first test, using the F198 M593 E64 line of PR8 virus, the results obtained were spotty. That these results were not due to technical factors was revealed by repeat test with similar findings. The spottiness of the results was surprising, in view of the clear-cut data that had been obtained in other tests (See Table I). In all previous tests the virus used in the neutralization test was the same, with respect to passage-line, as the antigen in the material used for the immunization of mice. The possibility was considered, therefore, that the PR8 virus present in the NIH control vaccine was different, in antigenic structure, from the PR8 virus used as antigen in the virus-neutralization test. On the supposition that the PR8 virus in the F2 vaccine was prepared from an egg-passage line not far removed from the mouse line, the regular mouse passage virus maintained in this laboratory was transferred to the egg for the preparation of antigen to be used in a repeat test for antibody. The results obtained with the F198 M720 E2 antigen were quite sharp, and supported the suspicion that the virus present in the NIH control vaccine was more closely related to the mouse-passage line than to the egg-passage line first used as the test antigen. The absence of any neutralizing effect against the F198 M70 TC717 E101 line of the PR8 strain indicates still greater differences between this agent and the PR8 virus in the vaccine.

These results are examples of the high degree of specificity of the antibody response to a single inoculation of influenza virus in experimental animals,[12] and of the variations in antigenic[13] and other properties[14] that can occur in different passage-lines of the same strain. The spotty results obtained with the F198 M593 E64 antigen suggests that antibody to this virus was induced by the inoculation of the 1:10 to 1:640 dilutions of the NIH control vaccine, but that the actual level of antibody for this line was much lower than for the F198 M720 E2 line. In this experiment there was no demonstrable crossing with the F198 M70 TC717 E101 line, although in other studies the relationships between all three have been demonstrated.

The results of this experiment emphasize the need for maintaining the identity of the test virus and the virus in the vaccine in any

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[10] Hirst, G. K., *J. Exp. Med.*, 1942, **76**, 195.

[11] Francis, T., Jr., and Salk, J. E., *Science*, 1942, **96**, 499.

[12] Magill, T. P., and Francis, T., Jr., *Brit. J. Exp. Path.*, 1938, **19**, 273.

[13] Francis, T., Jr., PROC. SOC. EXP. BIOL. AND MED., in press.

[14] Salk, J. E., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 134, 140.

TABLE I.

Results of Test for Antibody in Serum of Mice Inoculated with Serial Dilutions of 3 Different Preparations Containing the PR8 Strain (F198 M593 E60) of Influenza Virus Type A.

	Serum obtained from immunized mice (groups of 10)	Vaccine preparations 1	2	3
Dilutions of vaccine inoculated	64			0 0 0 0
	128			0 0 0 0
	256	0 0 0 0	0 0 0 0	0+++
	512	0 0 0 0	0 0 0 0	++++
	1024	0 0 0 0	0 0 0+	
	2048	0 0 0 0	++++	
	4096	0 0 0 0	++++	
	8192	++++		
	16000	++++		
	Normal serum plus virus	++++		

The symbols, + or 0, indicate the presence or absence of influenza virus infection in each of 4 embryos inoculated with the same serum-virus mixture. Evidence of infection was the presence of hemagglutinin in the allantoic fluid. The serum-virus mixture was made up of equal volumes of undiluted serum and 10–5 dilution of virus. The result of the virus titration was:

10–6	10–7	10–8	10–9
+++++ 0	++++++	++ 0 0 0 0	0 0 0 0 0 0

TABLE II.

Effect of the Quantity of Virus, Used in the Neutralization Test Upon the Titer of Neutralizing Antibody in Serum.

*Serum	Dilution	†Virus dilutions used in neutralization test 10–4	10–5	10–6	10–7	
Low titer	8	0 0 0 0	0 0 0 0			
	16	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	
	32	0 0 0+	0 0 0+	0 0 0 0	0 0 0 0	†Virus
	64	++++	++++	0 0++	0 0 0+	titration
	128			++++	0+++	10–4 ++++
						10–5 ++++
High titer	128	0 0 0 0	0 0 0 0			10–6 ++++
	256	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	10–7 ++++
	512	++++	0 0++	0 0 0 0	0 0 0 0	10–8 + 0 0 0
	1024	++++	++++	++++	0 0 0+	10–9 0 0 0 0
	2048			++++	0 0 0+	
Normal control	2	++++	++++	++++	++++	

Symbols same as in Table I.

* Low titer serum was pool from 10 mice inoculated 2 weeks earlier with 0.5 cc of 1:1024 dilution of allantoic fluid containing PR8 strain of influenza virus Type A.

High titer serum was from similar group inoculated with a 1:4 dilution of the same fluid.

† Virus used was in allantoic fluid from chick embryos infected with the same strain of virus used for immunization of mice, *i.e.*, PR8 (F198 M593 E64).

method employing active immunization for the estimation of the quantity of antigenic substance in a vaccine. Otherwise, the factor of qualitative differences among different lines of the same strain tend to confuse and obscure the quantitative estimate which the test is designed to provide. For this reason it is not known to what extent the data shown in Table V represent a correct evaluation of the antigenic extinction titer of any of the strains in the vaccine tested.

5. *Influence of age of mice upon the results of the antigenic extinction test.* It was of interest to determine to what extent age or size of mice influenced the results of the antigenic extinction test. Accordingly, 2 groups of mice of different ages were obtained from the same breeder. The weight of the young mice averaged 10 g and the older mice weighed about 26 g each. Groups of

TABLE III.

Results of Egg-Infectivity Titrations of Different Strains of Virus Used as Antigen in the Virus Neutralization Tests; to Illustrate the Degree of Stability of Infectivity of the Preparation when Stored at 4°C for Different Lengths of Time.

Strain Passage Date of preparation	Date of test	Infectivity titrations					
		Dilutions					
		10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
PR8 (11-28-46)	12-15-46		6/6	6/6	6/6	0/6	
F198	1- 2-47		6/6	6/6	5/6	0/6	
M593	31		6/6	6/6	3/6	0/6	
E64	2-13		6/6	6/6	4/6	1/6	
	27		6/6	6/6	2/6	0/6	
	3-14		6/6	6/6	2/6	0/6	
	4-11		4/4	2/4	0/4	0/4	
	21		4/4	2/4	0/4	0/4	
	5- 5		4/4	2/4	0/4	0/4	
PR8 (12-21-46)	12-27-46		4/4	4/4	4/4	3/4	1/4
F198	1-23-47		4/4	4/4	4/4	2/4	0/4
M70	31		6/6	6/6	5/6	0/6	0/6
TC717	3- 3		6/6	6/6	3/6	0/6	0/6
E101	5- 5		4/4	4/4	3/4	0/4	0/4
PR8 (2-17-47)	2-19-47		4/4	4/4	3/4	1/4	
F198	3-10		4/4	2/4	1/4	0/4	
M720	31		4/4	2/4	0/4	0/4	
E2	4-21		4/4	3/4	0/4	0/4	
	5- 5		3/4	0/4	0/4	0/4	
Weiss (12-9-46)	12-24-46	6/6	6/6	6/6	3/6	0/6	
F3	1- 8-47	6/6	6/6	4/5	3/6	0/6	
M32	2- 5	6/6	6/6	4/6	0/6	0/6	
E58	4- 3	4/4	0/4	0/4	0/4		
	21	2/4	1/4	0/4	0/4		
E59 (4-28-47)	28	4/4	4/4	1/4	1/4	0/4	
Lee (11-28-47)	12- 4-46	6/6	6/6	5/6	0/6	0/6	
F8	19	4/4	4/4	3/4	0/4	0/4	
M137	1-20-47	5/6	1/6	0/6	0/6		
E109	2-10	2/6	1/6	0/6	0/6		
E110 (2-12-47)	14	4/4	4/4	4/4	2/4	0/4	
	3-21	5/6	3/6	0/6	0/6		
	4-11	4/4	0/4	0/4	0/4		
	30	4/4	2/4	0/4	0/4		
E111 (4-28-47)	5- 5	4/4	4/4	1/4	0/4		

Denominator = No. of eggs inoculated.

Numerator = No. of eggs infected as indicated by presence of hemagglutinin in allantoic fluid.

10 mice were given one intraperitoneal inoculation of 0.5 cc of the respective 2-fold dilutions of the Provisional Control Vaccine of the National Institute of Health (Lot F2). Two weeks later all mice were bled and the sera tested for the presence of neutralizing antibody. The results are summarized in Table VI. The data for the old mice are the same as those shown in Table V. It is of interest that the antigenic extinction titration done in the older mice gave somewhat higher values than the test done in the younger mice. The earlier comments with regard to differences in results observed when different strains or passage-lines were used as antigen in the neutralization test apply to the present findings as well. Unfortunately, sufficient serum from the young mice was not available to permit further studies of the height to which antibody titers were elevated in the old and young mice inoculated with corresponding dilutions of the vaccine.

Summary. A procedure applicable to the standardization of influenza virus vaccines has

TABLE IV.

Influence of the Interval Between Inoculation and Bleeding of Mice upon Antibody Titer (as Measured by Virus-Neutralization in Chick Embryos and by Red Cell Agglutination-Inhibition).

Results of Virus-Neutralization Test† in Chick Embryos

Interval between inoculation* and bleeding (days)	Serum dilutions 4	16	64	256	1024	4096
7	0 0 0 0	0+++	++++	++++	++++	++++
10	0 0 0 0	0 0 0 0	++++	++++	++++	++++
14	0 0 0 0	0 0 0 0	0 0 0+	0+++	++++	++++
21	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0++	++++
Normal	++++					

(Symbols as in Table I.)

Results of Agglutination-Inhibition Test‡

Interval (days)	Serum dilutions 8	16	32	64	128	256	512	1024
7	0	0	0	0	0	0	+	+
10	0	0	0	0	0	0	+	+
14	0	0	0	0	0	0	+	+
21	0	0	0	0	0	0	+	+
Normal	+	+	+	+	+	+	+	+

* Mice inoculated intraperitoneally with 0.5 cc of 1:25 dilution of formalinized allantoic fluid containing PR8 strain of influenza virus Type A(F198 M593 E60).

† For the neutralization test in chick embryos 1000 50% egg-infecting doses were used.

‡ For the agglutination-inhibition test, 4 hemagglutinating units of virus were used.

TABLE V.

Antigenic Extinction Titration of N.I.H. Provisional Control Vaccine (Lot F2).

Serum obtained from immunized mice (Groups of 10)	Strain and passage line of virus used as antigen for detection of neutralizing antibodies in serum of immunized mice. PR8			Weiss	Lee
	F198 M593 E64	F198 M720 E2	F198 M70 TC717 E101	F3 M32 E58	F8 M137 E110
Dilutions of vaccine inoculated					
10	0 0 0+	0 0 0 0	++++	0 0 0 0	0 0 0 0
20	0 0 0 0	0 0 0 0	++++	0 0 0 0	0 0 0 0
40	0 0 0 0	0 0 0 0	++++	0 0 0 0	0 0 0+
80	0 0++	0 0 0 0	++++	++++	0 0 0+
160	0 0 0 0	0 0 0 0	++++	0+++	++++
320	0 0 0+	0 0 0 0		++++	++++
640	0 0++	0 0 0 0		++++	
1280	++++	0 0 0 0			
2560	++++	++++			
5120	++++	++++			
Normal controls	++++	++++	++++	++++	++++

Symbols same as in Table I.

been described. The total time required for completion of the test can be as little as 16 days. The procedure, referred to as the antigenic extinction titration, determines the highest dilution of antigen capable of inducing the formation of demonstrable amount of virus-neutralizing antibody in the pooled serum of groups of 10 mice. The test for neutralizing antibody is carried out *in ovo* and may, therefore, be used in those instances in which the strain of virus that may be included in a vaccine has been adapted only to the chick embryo and is not pathogenic for mice.

TABLE VI.
Comparison of Results of Antigenic Extinction Titration of N.I.H. Control Vaccine in Young and Old Mice.

Dilutions of vaccine inoculated	Challenge Virus*					
	PR8 F198 M720 E2		PR8 F198 M593 E64		Weiss F3 M32 E58	
	Serum from		Serum from		Serum from	
	Old mice	Young mice	Old mice	Young mice	Old mice	Young mice
10	0 0 0 0	0 0 0 0	0 0 0+	0 0 0 0	0 0 0 0	0 0 0 0
20	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	++++
40	0 0 0 0	0 0 0 0	0 0 0 0	0 0++	0 0 0 0	0+++
80	0 0 0 0	0 0 0 0	0 0++	++++	++++	++++
160	0 0 0 0	0 0 0 0	0 0 0 0	++++	0+++	++++
320	0 0 0 0	0 0 0 0	0 0 0+	++++	++++	++++
640	0 0 0 0	0 0 0 0	0 0++	0+++	++++	++++
1280	0 0 0 0	0+++	++++	++++	++++	++++
2560	++++	++++	++++	++++	++++	++++
5120	++++	++++	++++	++++	++++	++++
Controls	++++	++++	++++	++++	++++	++++

Symbols same as Table I.
* Approximately 1000 50% egg-infecting doses of each strain were used.

The use of chick embryos rather than mice as the indicator medium for virus neutralization offers the advantages of completion in 2 days rather than 10 days, applicability to egg-adapted strains and sharpness of results, as well as being less costly.

The importance of maintaining the identity of virus in vaccine and virus used as test antigen for measuring the antibody response induced by immunization has been illustrated and discussed.

15959

Experimental Infection of Domestic Animals with Japanese B Encephalitis Virus.*

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During the summer of 1946 a series of experiments were carried out on Okinawa with the general purpose of investigating the part that domestic animals might play in the epidemiology of Japanese B encephalitis.[1] Workers from Naval Medical Research Unit Number 2 had presented evidence that horses might have such a role.[2] It appeared desirable to obtain additional evidence on this point and also to study the other domestic animals which were common on the island during the epidemic of 1945. The experiments were designed (1) to determine how long virus persisted in the blood of experimentally infected animals, (2) to determine whether the virus was pathogenic for any of the species studied and (3) to study the time of appearance and increase of neutralizing and complement-fixing antibodies.

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[1] Thomas, L., and Peck, J. L., U. S. Naval Medical Research Unit No. 2, Official Report, 1945, unpublished.

[2] Hodes, H. L., Thomas, L., and Peck, J. L., Proc. Soc. Exp. Biol. and Med., 1945, **60**, 220.

Materials and Methods. Laboratory Facilities and Animals. A field laboratory was set up in a quonset hut of the former Military Government Hospital at Goya. An adequately screened adjoining building housed the smaller animals, while horses were kept in an especially constructed stable designed by previous workers.[2] Swiss albino mice 1 to 3 months of age were used for passage and isolation of virus. These animals were in good condition on arrival, but suffered, to some extent, from intercurrent infections during the period of the experiments.

Virus Strain. The Okinawan strain of Japanese encephalitis, isolated by Thomas and Peck,[3] was used in all experiments. This strain had been through from 13 to 15 mouse brain passages. Brains were stored, during early experiments, in the freezing compartment of an icebox and later in an icebox at a temperature between 4 and 10°C.

Route of Infection and Dose of Virus. All animals were infected by the intravenous route with 1.0 ml of a 10^{-2} dilution of a fresh mouse brain suspension. 10% normal rabbit serum saline was used as diluent. Concurrent titrations were not done, but subsequent intracerebral titrations in mice suggested that the titer of these brains was of the order of 10^{-7}.

Experimental Animals. Pigs, goats, and chickens were obtained from the south central part of Okinawa. The pigs were estimated to be 4 months old, the goats 4 to 8 months, and the chickens less than a year old. Domestic Muscovy ducks were obtained from the village of Hentona and were at least 2 years old. Young horses could not be found on the main island and, for this reason, were procured on the small island of Iheya Shima off the northern tip of Okinawa where much of the horse-breeding has been carried on in recent years. Three were colts 2 to 4 months of age, and the fourth was a 2-year-old.

Recovery of Virus from Blood. Animals were bled before inoculation and 1, 3, 6, 9, and 12 days after inoculation. After the blood had coagulated, 0.03 ml of the serum with a small quantity of suspended cells was injected intracerebrally into each of 3 or 4 mice. Deaths occurring between the 4th and 10th days with characteristic signs and absence of other apparent illness were considered to be probably due to the encephalitis virus. However, in order to better establish this point, brains of moribund or recently dead mice were harvested, stored, and later transported to Berkeley for definite identification by passage and neutralization with specific antiserum. Virus was still present in many of the brains after 6 weeks of storage at ordinary icebox temperatures, but may have been lost from others.

Neutralization Tests. Serial 10-fold dilutions of infected mouse brain suspensions in 10% normal rabbit serum broth were mixed with equal amounts of undiluted, noninactivated, test serum. After incubation for 2 hours at room temperature, the serum-virus mixture was inoculated intracerebrally in 0.03 ml amounts into groups of mice 2 to 6 weeks old. The LD_{50} of the virus-suspension was determined by titration under similar conditions with normal rabbit serum and the neutralizing capacity of the test serum expressed in terms of a neutralization index. Test mice were observed for 14 days.

Complement-fixation Tests. Mouse brain antigens were prepared by the method of Casals and Palacios.[4] Sera were inactivated for 30 minutes at 60° or at 65°C, if necessary. At least 2 other mouse brain antigens were run concurrently with each serum. Overnight fixation at 4°C was used. Results were expressed in terms of the original serum dilution.

Results. Recovery of Virus from Blood. The results of experiments with each species are summarized in Table I. Virus was recovered from all the pigs and ducks 24 hours after inoculation, in sufficient amounts to kill all tested mice within 7 days. Smaller amounts appeared to be present in the blood

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[3] Casals, J., and Palacios, R., *J. Exp. Med.*, 1941, **74**, 409.

[4] Hammon, W. McD., Reeves, W. C., and Burroughs, R., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 304.

TABLE I.
Results of Intracerebral Inoculation of Mice with the Blood of Experimental Animals.

			Days after inoculation				
Species	No.	Pre-inoc.	1	3	6	9	12
Horse	1	2/3*	0/4	0/3	0/3	0/3	0/3
	2	1/3	0/4	0/3	0/3	0/3	0/3
	3	1/3	0/4	0/3	0/3	0/3	0/3
	4	0/3	1/4	0/3	0/3	0/3	0/3
Goat	1	0/2	1/3	1/3	0/4	0/4	0/4
	2	0/2	0/3	0/3	0/4	0/4	0/4
	3	0/3	0/3	0/3	0/4	0/4	0/4
	4	0/3	0/3	0/3	0/4	0/4	0/4
Pig	1	0/2	3/3†	0/3	(dead)		
	2	2/2	3/3†	0/4	0/4	0/4	—
	3	0/2	3/3†	0/3	0/4	(dead)	
Duck	1	1/2	3/3†	1/4	0/4	1/4	—
	2	0/2	3/3†	0/4	1/4	1/4	—
	3	1/2	3/3†	0/4	0/4	1/3	—
	4	0/2	3/3†	0/4	0/4	0/3	—
Chicken	1	0/3	1/4	1/4	0/3	0/2	0/3
	2	1/3	1/4	3/4	0/3	0/2	0/3
	3	0/3	1/4	2/3	0/3	0/2	0/3
	4	0/3	3/4	2/4	0/3	1/2	0/3

* Dead/Total.

† Specificity of deaths confirmed by passage and/or neutralization.

of chicken No. 4 on the 1st and 3rd days and of chickens No. 2 and No. 3 on the 3rd day. Minimal amounts may have been recovered from horse No. 1 on the day following inoculation and from goat No. 1 on the 1st and 3rd days and in certain of the later duck bloods, but the specificity of these deaths was not established. It seems certain that, if virus was present, the amount was small.

Evidence of Illness. None of the goats or ducks appeared ill at any time. One of the chickens appeared listless 5 days after inoculation but soon recovered. The 4 horses likewise showed no rise in temperature or evidence of illness. The 3 young colts were subsequently shown to have high levels of antibody before inoculation, and presumably only the 2-year-old (horse No. 4) was nonimmune.

A very different picture was observed with the pigs. All 3 appeared ill on the 5th day, with ataxia, drowsiness, irritability, anorexia, and conjunctivitis the most notable findings. Temperatures ranged from 103^2 to 106. Pigs No. 1 and No. 3 became progressively worse and died on the 6th and 8th days, respectively. Pig. No. 2 made a gradual recovery, but remained ataxic and walked with a peculiar, high-stepping gait. Complete autopsies were done on both animals from 4 to 8 hours after death. There were no grossly abnormal findings other than diffuse hyperemia of the brain in each case and cerebellar necrosis in pig No. 1. The cisternal fluid of pig No. 1 contained more than 500 leucocytes. Suspensions of various parts of both brains were inoculated into mice, but virus was not recovered.

Neutralizing and Complement-fixing Antibodies. The results of serial determination of neutralizing antibody levels are presented in Table II, and those of complement-fixing antibody levels in Table III. The single susceptible horse showed definite evidence of neutralizing antibody on the 6th day, with a further rise on the 12th day. All 4 goats had antibody by the 6th day and failed to rise further during the following 18 days. Pigs No. 2 and No. 3 also had antibody on the 6th day, the day before the latter died. The response in the ducks was similar. Two of the 4 chickens neutralized strongly on the

TABLE II.
Serial Determinations of Intracerebral Neutralizing Antibody in Experimental Animals.

Species	No.	Pre-inoc.	Days after inoculation					
			1	3	6	9	12	24-28
Horse	1	+++*	+++	+++	+++	++	+++	
	2	+++		+++	+++	++	+++	
	3	++	+	+	+	+	++	
	4	0	0	±	±	+	+	
Goat	1	0	0	0	+	+	+	+
	2	0	0	±	+	+	+	+
	3	0	0	0	+	+	+	+
	4	0	0	0	+	+	+	±
Pig	1	0	0	±	(dead)			
	2	0	0	±	+	++		++
	3	0	0	0	+	(dead)		
Duck	1	0	0	0	+	0		++
	2	0	0	±	±			++
	3	0	0	0	+	+		++
	4	0		0	±	+		++
Chicken	1	0			0		±	
	2	0			++		+++	
	3	0			±		+	
	4	0			++		++	

* Graded according to neutralization index: 0 = 0 LD_{50}; ± = 10-32 LD_{50}; + = 32-100 LD_{50}; ++ = 100-320 LD_{50}; +++ = 320-1000 LD_{50}.

TABLE III.
Serial Determination of Complement-fixing Antibody Titers of Experimental Animals.

Species	No.	Pre-inoc.	Days after inoculation					
			1	3	6	9	12	28
Horse	1*	8	8	8		8	8	
	2*	32	8	32	64	32	16	
	3*	4	8	4	4	4	4	
	4	4	8	8	8	32	32	
Goat	1	8	16	16	8	16	16	16
	2	0	0	8 (?)	0	0	0	32
	3	0	0	4		8		16
	4	0	0			8	4	64
Pig	1	0	0	0 (Dead)				
	2	0	0					16
	3	4	0	0 (Dead)				

* Showed neutralizing antibody in pre-inoculation serum.

6th day, and on the 12th day these 2 had higher neutralization indices than any of the other species tested. The proportionately greater inoculum in these small birds may have contributed to this difference.

Complement-fixation tests were performed only with the sera of the mammals. Three animals which had shown no neutralizing antibody before inoculation showed fixation in their initial bleedings in dilutions of 1:4 and 1:8. This may represent specific antibody persisting after the disappearance of neutralizing antibody, for sera from the same specie of animals bled in New Caledonia uniformly failed to show fixation even at a dilution o 1:4. In horse No. 4 the titer rose rapidly by the 9th day and had reached 64 on the 12th day, at which time the neutralization index was still relatively low. The titer o one goat began to rise on the 9th, of an other on the 12th day, while that of a third did not show a rise until the last bleeding. The fourth goat failed to show a significant rise. The single surviving pig had a titer of only 16 in his latest bleeding, 28 days after inoculation.

Discussion. When the data obtained in these experiments is considered along with the earlier horse experiment of Thomas and Peck[2] and the chicken experiments of Hammon *et al.*,[5] it appears that only one of the species so far tested, namely the goat, can reasonably be excluded from consideration as a possible source of infection for the insect vectors of this virus. There is no doubt that experiments of this sort leave much to be desired, since both the route and dosage of virus may differ greatly from those occurring in the natural infection. There obviously remains a broad field for investigation before this question can be settled.

The usefulness of the complement-fixation test, stressed by other workers,[4,3] is further emphasized by this animal data. Complement-fixing antibody appeared as early as neutralizing antibody and its titer then rose sharply, whereas the titer of the latter remained at relatively low levels during the period of observation. This relationship is very different from that seen in experimental infection of horses with Western equine encephalomyelitis virus, in which the neutralizing antibody appears earlier and rapidly reaches a high level.

The finding of antibody in colts 2 to 4 months of age is of some interest. These animals had been foaled during late winter or early spring seasons and were procured at a time before any clinical encephalitis had appeared. It seems, therefore, unlikely that they had acquired antibody as a result of infection. Furthermore, their dams, along with 6 other horses, had been bled on Iheya Shima, and all showed high levels of neutralizing antibody. The data point toward maternal transmission as the probable explanation of antibody in the colts.

The observation that all 3 inoculated swine developed signs of encephalitis and that 2 of the 3 died is of considerable interest. Despite the fact that virus was recovered from the blood of each animal on the day following injection, the failure to recover virus from the brains makes it impossible to conclude that the experimental agent was responsible for their illnesses or deaths. Intercurrent infection by some agent pathogenic for swine could not be excluded even though (a) the animals had been observed in isolation from other porcine contacts for more than 10 days before any showed signs of illness; (b) the clinical picture suggested an infection of the central nervous system and the autopsies showed no pathological findings outside the central nervous system; and (c) a fourth pig, which was kept in the same pen throughout the experiments but not inoculated with virus, showed no sign of illness. It is obvious, however, that the pathogenicity of this virus for swine can be established only by further experiments. The epidemiological and veterinary implications of this possibility appear to warrant investigation.

Summary. 1. Okinawan horses, goats, pigs, ducks, and chickens were inoculated intravenously with the virus of Japanese B encephalitis in order to obtain additional data regarding their role as natural reservoirs of disease.

2. Virus was recovered from the blood of pigs and ducks 24 hours after intravenous inoculation and was probably present for a longer period in chickens.

3. Two of 3 inoculated pigs died with characteristic signs of encephalitis while the third recovered after a long illness of a similar nature. Virus was not recovered from the brains of the animals which died. The other species showed no clinical evidence of infection.

4. Complement-fixing antibodies in experimental animals rapidly reached high levels, whereas neutralizing antibody levels rose more slowly.

The authors wish to acknowledge the assistance of Commander Fred Butler, MC, USN, of Naval Medical Research Unit Number Two, Guam, of the members of the Military Government Staff on Okinawa,

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[5] Meiklejohn, G., and Dean, B., unpublished data.

in particular Lt. Commander John J. Halbert, MC, USNR, Capt. Bailey, VC, AUS, and Capt. Catlin, CAC, AUS, and of Lt. (JG) Williams, MC, USNR, and other members of Naval Medical Research Unit Number One, Berkeley. The facilities of the Virus Laboratory in Berkeley were generously made available by Dr. M. D. Eaton, Director, Virus Laboratory, California State Department of Public Health.

15960

Chemical Antagonism of Pteroylglutamic Acid in a Pig; Hematopoietic Effect of Extrinsic and Intrinsic Factors.*

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It has been established that pteroylglutamic (folic) acid (PGA) is concerned with the formation of various cells of the blood, particularly erythrocytes and granulocytes. The vitamin has been studied in several animal species, including chicken, turkey, rat, dog, and monkey, as well as in man.

In man, with various types of macrocytic anemia, including that seen in pernicious anemia and in sprue, a striking hematologic response is usually obtained when PGA is administered.[1,2,3,4] This response cannot be differentiated clearly from that characteristically produced by refined liver extracts,[†] or from that caused by the administration, in pernicious anemia in relapse, of normal gastric juice (containing intrinsic factor) together with a heat-stable substance (extrinsic factor) found in beef skeletal muscle, casein, and other natural materials.[5] The roles of the non-PGA antipernicious anemia (APA) factor of liver extracts and of the extrinsic factor have been most difficult to study, because the effects produced in man have not been demonstrable in animals, even in those with anemia or leucopenia induced by a deficiency of PGA.[6,7,8]

Only swine have offered promise for studies of this character. The liver of this species is rich in the APA factor and is widely employed in the manufacture of liver extracts used in the treatment of macrocytic anemias. It was shown by Miller and Rhoads[9] that, under certain dietary conditions, swine develop an anemia that responds to injections of liver extract. Most striking, however, was the report of Cartwright, Wintrobe and Hum-

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[*] This investigation was supported by a grant-in-aid from the United States Public Health Service. The chemical antagonist of PGA and a large proportion of the vitamins used in the purified diets were supplied by the Lederle Laboratories Division, American Cyanamid Company. The succinylsulfathiazole was generously supplied by the Medical Research Division, Sharp and Dohme, Inc. Crude sodium caseinate was donated by the Borden Company.

[1] Spies, T. D., Vilter, C. F., Koch, M. B., and Caldwell, M. H., *South. Med. J.*, 1945, **38**, 707.

[2] Darby, W. J., and Jones, E., Proc. Soc. Exp. Biol. and Med., 1945, **60**, 259.

[3] Moore, C. V., Bierbaum, O. S., Welch, A. D., and Wright, L. D., *J. Lab. Clin. Med.*, 1945, **30**, 1056.

[4] Welch, A. D., Heinle, R. W., Nelson, E. M., and Nelson, H. V., *J. Biol. Chem.*, 1946, **164**, 787.

[†] Refined liver extracts contain insignificant amounts of determinable PGA, often less than 0.001 mg per cc.

[5] Castle, W. B., *Harvey Lectures*, 1934-35, **30**, 37.

[6] Day, P. L., Langston, W. C., Darby, W. J., Wahlin, J. G., and Sims, V., *J. Exp. Med.*, 1940, **72**, 463.

[7] O'Dell, B. L., and Hogan, A. B., *J. Biol. Chem.*, 1943, **149**, 323.

[8] Stokstad, E. L. R., and Jukes, T. H., Proc. Soc. Exp. Biol. and Med., 1946, **62**, 112.

[9] Miller, D. K., and Rhoads, C. P., *J. Clin. Invest.*, 1935, **14**, 153.

[10] Cartwright, G. E., Wintrobe, M. M., and Humphreys, I., *J. Lab. Clin. Med.*, 1946, **31**, 423.

phreys[10] describing the effect in a weanling pig of a diet containing "Labco" vitamin-free casein (26.1%), sucrose, lard (11%), salts, succinylsulfathiazole (2%), choline chloride, inositol, thiamine, riboflavin, pyridoxine, nicotinic acid, calcium pantothenate, and *p*-aminobenzoic acid. This dietary regimen produced a severe depression of growth and marked hematologic as well as other changes. The volume of packed red blood cells was reduced to 21 cc from 50 cc per 100 cc of blood; the anemia was characterized as normocytic. After 120 days on the diet, intramuscular administration of biotin (1 mg per day) for 17 days caused no apparent improvement and 1 cc of purified liver extract was then administered by the intramuscular route daily for 10 days. A definite reticulocytosis (peak, 9.4% on tenth day) occurred and the hemoglobin concentration and the volume of packed red cells promptly increased. The growth response was striking, so that 50 days after the liver therapy the weight had doubled (from about 29 kg).

By analogy with results obtained in other species, it may be postulated that this pig developed a deficiency of PGA. In the rat, a comparable dietary regimen produces a deficiency that responds promptly to combined therapy with biotin and PGA.[11,12,13] In addition to deficiencies of these factors, a deficiency of the extrinsic factor also may have developed, since it has been shown by Castle *et al.*[14] that "Labco" vitamin-free casein contains little or none of this substance, as evidenced by tests in human patients. The possibility that deficiencies of PGA and of extrinsic factor can be corrected by the administration of materials found in refined extracts of liver, essentially free from PGA,[14] has important implications and is deserving of further study. Although the production of such a syndrome in swine offers a number of disadvantages, particularly that of expense, a program of research directed toward the isolation and study of antianemic factors would be facilitated greatly by the availability of an animal species in which a suitable deficiency could be produced. Accordingly, swine were placed on a diet closely resembling that of Cartwright *et al.*[10]

Experimental. Six Berkshire pigs 9 weeks of age were employed; the weights varied from 3.9 to 9.6 kg. One animal, (No. 1♀, 3.9 kg) was fed a commercial diet designed for the feeding of swine; the other animals were given a purified diet of the following composition: casein ("Labco" vitamin-free), 20 g; glucose ("Cerelose"), 48.7 g; hydrogenated vegetable oils ("Primex"), 18 g; corn oil, containing vitamins A, D, and E,[‡] 2 g; salt mixture (U.S.P. No. 2), 3.8 g; accessory salts,[§] 0.2 g; cellulose ("Cellu-flour"), 5 g; succinylsulfathiazole, 2 g; choline chloride, 0.2 g; inositol, 0.1 g; thiamine hydrochloride, 0.5 mg; riboflavin, 1 mg; pyridoxine hydrochloride, 0.5 mg; nicotinamide, 5 mg; calcium pantothenate, 5.5 mg; ascorbic acid, 10 mg; 2-methyl-1,4-naphthoquinone, 1 mg; biotin, 0.02 mg. In addition, two animals (No. 2♂, 8.5 kg, and No. 3♀, 6.9 kg) were given a daily oral supplement of synthetic PGA, 0.02 and 0.2 mg per kg, respectively. Pigs No. 4♂ (8.5 kg), No. 5♀ (9.6 kg), and No. 6♀ (7.3 kg) were given no PGA. After 16 days on this dietary regimen, the diet of pig No. 6 was supplemented with a chemical antagonist of PGA. This antagonist was used in the crude form, as described in the accompanying paper by Franklin, Stokstad and Jukes,[15] and was fed at a level of 0.1%[¶] of the diet.[**]

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[11] Black, S., McKibbin, J. M., and Elvehjem, C. A., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 308.

[12] Welch, A. D., *Fed. Proc.*, 1942, **1**, 171.

[13] Welch, A. D., and Wright, L. D., *J. Nutr.*, 1943, **25**, 555.

[14] Castle, W. B., Ross, J. B., Davidson, C. S., Burchenal, J. H., Fox, H. J., and Ham, T. H., *Science*, 1944, **100**, 81.

[‡] Two grams of the corn oil preparation contained vitamin A, about 4500 units; vitamin D, about 560 units; and mixed tocopherols, about 2.7 mg.

[§] Accessory salts contained in 0.2 g (expressed in mg): KCl, 100; NaCl, 87.2; $FeSO_4$, 10; $MnSO_4$, 1; $ZnSO_4 \cdot 7H_2O$, 1; $CuSO_4 \cdot 5H_2O$, 1; NaI, 0.3; NaF, 0.1; $COCl_2 \cdot 6H_2O$, 0.01.

[15] Franklin, A. L., Stokstad, E. L. R., and Jukes, T. H., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 368.

[¶] The crude antagonist was prepared by Drs. M. E. Hultquist and J. M. Smith, Jr., Calco Chemical Company, by reacting 2,4,5-triamino-6-hydroxy-

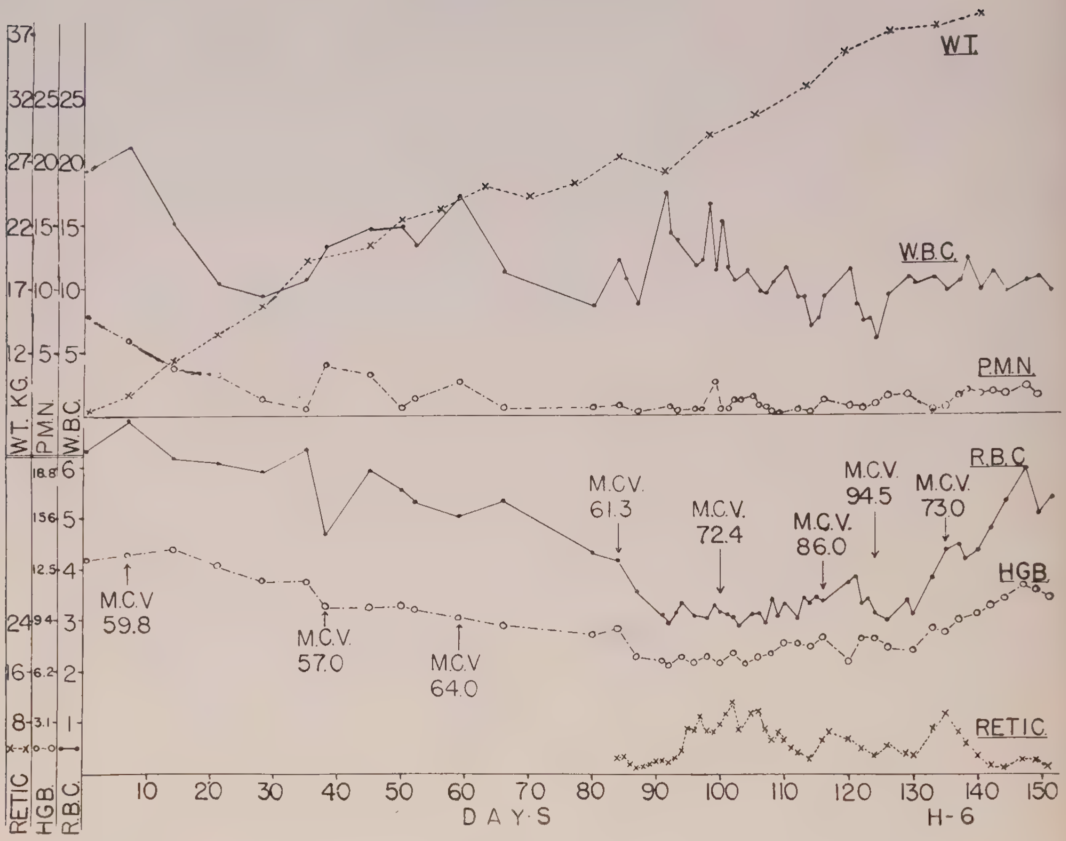

FIG. 1.

Hematologic and weight changes in a pig fed a purified diet containing 2% succinylsulfathiazole. After 16 days, the diet was supplemented with an antagonist of pteroylglutamic acid; this supplementation was continued throughout the experiment. On the 83rd day, and for a total of 10 days, normal human gastric juice was administered daily, together with an alcoholic extract of crude casein. For 14 days only, beginning on the 83rd day, the diet contained crude sodium caseinate, in place of vitamin-free casein ("Labco").

W.B.C. = White blood cells × 10^3.
P.M.N. = Polymorphonuclear leucocytes × 10^3.
HBG = Hemoglobin in g/100 cc.
R.B.C. = Red blood cells × 10^6.
RETIC = Reticulocytes in %.
M.C.V. = Mean corpuscular volume in cubic microns.

Results. In contrast to the striking result obtained by Cartwright *et al.*,[10] no evidence of any deficiency in growth or in hematopoiesis was observed in those swine given the highly purified diet containing succinylsulfathiazole, except in the case of the one animal also given the chemical antagonist. The reason for this difference in the findings of the two laboratories cannot be stated. Of possible significance were the omission of *p*-amino-

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pyrimidine and *p*-amino-benzoyl-1(+)-glutamic acid with 2,3-dibromobutyraldehyde in the reaction described by Angier, R. B., Boothe, J. H., Hutchings, B. L., Mowat, J. H., Semb, J., Stokstad, E. L. R., SubbaRow, Y., Waller, C. W., Cosulich, D. B., Fahrenbach, M. J., Hultquist, M. E., Kuh, E., Northey, E. H., Seeger, D. R., Sickels, J. P., and Smith, J. M., Jr., *Science*, 1946, **103**, 667. The percentage of crude antagonist in subsequent lots of diet was varied in proportion to the potency of the antagonist, as determined by Dr. E. L. R. Stokstad by microbiological assay using *S. faecalis R.*

** We are much indebted to these workers for supplying the antagonist and for the frequent discussions of their and our studies.

benzoic acid and the inclusion of biotin in our diet.

In the pig given the crude antagonist of PGA the growth rate gradually decreased and a state of severe anemia developed with the characteristics indicated in Fig. 1. A patchy alopecia appeared, but the hair-loss was not extensive. Profuse diarrhea was noted, the animal became listless and evidenced an unwillingness to stand, although no signs of neuromuscular degenerative changes were found. The appetite, which previously had been excellent, quite rapidly diminished to such a degree that the caloric intake became critically low (minimal daily caloric intake, about 18 calories per kg), and the continued survival of the animal became doubtful.

Therapy. At this time the vitamin-free casein of the diet was withdrawn and replaced by crude sodium caseinate in equivalent amount. Because of the low food intake, the pig was given in addition, by daily gastric intubation, an extract of crude casein in an amount equivalent to about 100 g of casein, together with from 80 to 150 cc of fresh neutralized human gastric juice. Since alcohol-extracted casein has been shown by Castle *et al.*[14] to be essentially free of extrinsic factor, we employed an extract of casein prepared by exhaustive treatment of crude casein with ethanol (95%) at 140°C;[††] the extract was then concentrated and extracted with petroleum ether to remove fats and contaminating oils. The sodium caseinate substituted for the purified casein, and an alcoholic extract of crude casein entirely comparable to that used in the pig, contained extrinsic factor, as demonstrated by tests in patients with pernicious anemia in relapse. None of the materials administered contained appreciable amounts of microbiologically determinable PGA.[‡‡]

After a total of 10 days of supplementation by gastric intubation and 14 days of feeding sodium caseinate in the diet, the pig was returned to the highly purified diet. As a result of the supplementation, improvement in appetite and vigor were rapid and unmistakable. Within 10 days after supplementation was begun the appetite improved and the animal was stronger and much more alert. The stools, previously very diarrheic, assumed a semi-solid consistency, although they were still of a greenish-black color. Within another week the animal appeared to be normal with respect to appetite, alertness and strength. On the eleventh day after supplementation was begun, the reticulocyte count began to increase; 3 days later the level was 9% and a peak of 11% was attained after an additional 5 days. Other hematologic data are shown in Fig. 1.

It is to be noted that the improvement in appetite, growth and hematopoiesis, initiated by the supplementation, has continued unabated to the present date, approximately 10 weeks from the cessation of therapy, despite the continued administration of a purified diet, succinylsulfathiazole, and the antagonist of folic acid.

Discussion. As has been mentioned previously, no adequate explanation can be offered to account for the failure to obtain results comparable to that of Cartwright, Wintrobe and Humphreys,[10] when the purified diet free from the antagonist of PGA was used. Possibly the inclusion of biotin in our diet may account for the different result; for example, a facilitation of the synthesis of PGA may have occurred. In any case, the inclusion of the chemical antagonist of PGA in the same diet severely depressed growth and the formation of erythrocytes and leucocytes.

It cannot now be stated that the response of the pig to the administration of a crude source of extrinsic factor, together with normal human gastric juice, was due to the combined effect of the two materials. A preliminary trial of the casein extract alone was prevented by the severity of the syndrome that developed. It is unlikely that gastric juice would have been efficacious by itself, since the studies of Castle and his associates have shown adequately that intrinsic factor alone produces no significant hematopoietic effect in human patients with pernicious anemia in relapse. Whether the pig had developed a deficiency of intrinsic factor, of course, cannot be stated, although a gastric analysis after stimulation with histamine, about 3 weeks prior to the initiation of therapy, indicated a high total acidity with absence of free hydrochloric acid. Studies designed to answer some of these questions are now in progress.

[††] The alcoholic extract of crude casein was generously supplied by the Nutritional Biochemicals Corporation, Cleveland, Ohio.

[‡‡] The samples of gastric juice administered were not analyzed for PGA; however, no sample so far tested has contained more than 2 μg of PGA/100 cc. Microbiologic assay of the sodium caseinate, after tryptic digestion and treatment with hog kidney conjugase, indicated a PGA content of 1.6 μg/g; the alcoholic extract of casein supplied not more than 0.1 μg of PGA daily.

Summary. Interference with the metabolism of pteroylglutamic acid in the pig, through the use of a crude chemical antagonist, interrupts growth and significantly inhibits the formation of erythrocytes and of granulocytes. This interference is removed, despite continued feeding of the antagonist, by administration of a crude source of extrinsic factor (essentially free of PGA), together with normal human gastric juice. This finding affords an experimental animal with which to study the mechanism of action of antianemic substances and their functional relation to folic acid; also, a suitable bioassay tool is offered for guiding the isolation of antianemic factors of unknown chemical composition.

The administration of a purified diet similar to that successfully used by Cartwright *et al.*[10] failed to produce a failure in growth and in hematopoiesis in swine. It is suggested that this failure may possibly be attributable to the presence of biotin in the diet employed in this study.

15961

Acceleration of Pteroylglutamic Acid Deficiency in Mice and Chicks by a Chemical Antagonist.

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In another communication[1] the effect on rats of a synthetic preparation "antagonistic" to pteroylglutamic acid was described. The present article describes the results obtained with mice and with chicks which received the same preparation. An acceleration of pteroylglutamic acid deficiency was observed, and the effect was prevented by adding pteroylglutamic acid to the diet at levels sufficient to overcome the action of the antagonistic preparation.

Experimental. Rockland female mice, 5 to 6 weeks old, were kept in wire-floored cages. Five animals were used per group. The following basal diet was fed (diet 1) glucose (Cerelose), 72 g; washed casein (Labco), 20 g; salt mixture,[2] 4 g; corn oil (Mazola) *plus* vitamins A, D and E, 3 g; succinylsulfathiazole, 1 g; choline chloride, 0.1 g; inositol, 0.1 g; niacinamide, 5 mg; calcium pantothenate, 5 mg; thiamine HCl, 1 mg; riboflavin, 1 mg; pyridoxine HCl, 1 mg; *p*-aminobenzoic acid, 1 mg; 1-acetoxy-2-methyl-4-naphthyl sodium phosphate, 0.5 mg; biotin, 0.02 mg. Three grams of the corn oil preparation contained vitamin A (acetate), 1500 U.S.P. units; vitamin D (Delsterol), 200 A.O.A.C. chick units; mixed tocopherols, 34 mg.

The antagonist was prepared by condensing 2,4,5-triamino-6-hydroxypyrimidine and *p*-aminobenzoyl-*l*(+)-glutamic acid with 2,3-dibromobutyraldehyde in the reaction described elsewhere.[3] The preparation was carried out by Dr. M. E. Hultquist and Dr. J. M. Smith, Jr.* The reaction product was used without purification. A similar product, using *p*-aminobenzoyl-*d*(—)-glutamic acid has been stated to have "displacing" activity for pteroylglutamic acid in the growth

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[1] Franklin, A. L., Stokstad, E. L. R., Belt, M., and Jukes, T. H., *J. Biol. Chem.*, 1947, in press.

[2] Hawk, P. B., and Oser, B. L., *Science*, 1931, **74**, 369.

TABLE I.
Growth of Mice on Diet 1 as Affected by Various Supplements.

Group No.	Supplement per kilo of diet 1	Body wt (g)						
		0	1	2	3	4	5	6 wk
1	None	15	18	21	21	23	24	24
2	10 g crude antagonist	16	17	19	19	17	16	16
3	10 g crude antagonist + 0.1 g pteroylglutamic acid	16	20	22	22	26	25	26

TABLE II.
Hematological Observations with Mice Receiving Diets Described in Table I.

Group No.	Hemoglobin, g per 100 cc		White cells ($\times 10^3$), per mm^3		Differential count at 4 wk, %*			
	at 4 wk	at 6 wk	at 4 wk	at 6 wk	N	L	M	E
1	16.9	17.6	9.3	12.1	24	73	2	0 to 1
2	15.9	13.0	3.3	3.3	21	77	1	0
3	17.4	18.5	10.1	12.6	23	76	0 to 1	0 to 1

* N = Neutrophils; L = Lymphocytes; M = Monocytes; E = Eosinophils.

of *S. faecalis* R.[4]

The supplements fed are described in Table I. The mice on the basal diet (Group 1) did not develop any signs of deficiency when the experiment was terminated at the end of 6 weeks. At this time the 2 surviving animals in Group 2, which received the antagonist, were in a moribund condition. The animals in this group were emaciated, but the chromodacryorrhea and very ruffled fur which were observed in rats on a similar diet[1] were not found. When the 2 surviving mice in Group 2 were killed and autopsied, the mouths were normal in contrast to the results with rats. The livers were yellow, but appeared normal in size and texture. The uteri were atrophic. Other data are summarized in Tables I and II, which show that complete protection was given by pteroylglutamic acid. The data in Table II indicate that the formation of white cells of both the myeloid and lymphoid series was depressed equally by the antagonist. In this respect the mice differed from rats, in which species the reduction of the granulocyte count is more conspicuous than the reduction of the lymphocyte count in pteroylglutamic acid deficiency.[1,5]

In experiments with chicks the basal diet had the following composition: Glucose (Cerelose), 58.5 g; purified casein (Labco), 20 g; gelatin, 8 g; calcium gluconate, 5 g; cystine, 0.4 g; choline chloride, 0.2 g; inositol, 0.1 g; bone ash, 2 g; NaCl, 0.6 g; KH_2PO_4, 0.45 g; K_2HPO_4, 0.6 g; $MgSO_4$, 0.25 g; $MnSO_4 \cdot 4H_2O$, 0.05 g; ferric citrate, 0.05 g; $CuSO_4 \cdot 5H_2O$, 2 mg; $Al_2(SO_4)_3 \cdot 18H_2O$, 1.6 mg; zinc acetate, 1.4 mg; KI, 0.6 mg; cobalt chloride, 0.4 mg; nickel chloride, 0.2 mg; calcium pantothenate, 5 mg; niacinamide, 5 mg; riboflavin, 1 mg; pyridoxine HCl, 1 mg; thiamine HCl, 1 mg; *p*-aminobenzoic acid, 1 mg; 1-acetoxy-2 methyl-4-naphthyl sodium phosphate, 0.5 mg; biotin, .02 mg; to which were added vitamin A (acetate), 1500 U.S.P. units; vitamin D, 200 A.O.A.C. units; mixed tocopherols, 34 mg dissolved in corn oil (Mazola) to a total of 3 g. Day-old New Hampshire chicks were placed on the diets. The usual signs of pteroylglutamic acid deficiency were

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[3] Angier, R. B., Boothe, J. H., Hutchings, B. L., Mowat, J. H., Semb, J., Stokstad, E. L. R., SubbaRow, Y., Waller, C. W., Cosulich, D. B., Fahrenbach, M. J., Hultquist, M. E., Kuh, E., Northey, E. H., Seeger, D. R., Sickels, J. P., and Smith, J. M., Jr., *Science*, 1946, **103**, 667.

[*] Calco Chemical Division, American Cyanamid Company, Bound Brook, N.J.

[4] Martin, G. J., Tolman, L., and Moss, J., *Arch. Biochem.*, 1947, **12**, 318.

[5] Spicer, S. S., Daft, F. S., Sebrell, W. H., and Ashburn, L. L., *U. S. Pub. Health Rep.*, 1942, **57**, 1559.

TABLE III.
Effect of Various Additions to a Basal Purified Diet, Deficient in Pteroylglutamic Acid (PGA), on Growth and Hemoglobin Formation in Chicks.

			Weights and number of survivors (in parentheses) at various ages			
Experiment No.	Group	Supplement per kilo of diet	1 day g	14 days g	28 days g	Hemoglobin, 28 days, g%
1	1	None	46 (11)	91	116 (11)	6.3
1	2	0.1 mg PGA	44 (10)	102	177 (10)	8.2
1	3	0.1 ” ” + 1 g antagonist	45 (6)	85 (6)	95 (2)	4.7
1	4	10 ” ” + 1 ” ”	45 (6)	145	353 (6)	9.6
1	5	1 ” ”	44 (10)	127	310 (10)	10.0
2	6	None	38 (10)	75 (9)	80 (2)	
2	7	10 g antagonist	36 (5)	57 (4)	—*	
2	8	0.1 mg PGA	40 (10)	81 (8)	167 (5)	
2	9	0.3 ” ”	39 (10)	100	253 (10)	
3	10	None	43 (10)	95 (10)	166 (7)	7.7
3	11	1 mg PGA	45 (10)	148 (9)	321 (9)	9.1
3	12	1 ” ” + 10 g antagonist	43 (10)	96 (8)	106 (3)	8.4

* All dead by three weeks.

TABLE IV.
Observations on Red and White Cell Counts in Chicks in Experiment 3 as Affected by PGA Deficiency.

			Erythrocyte count cells per mm^3 ($\times 10^6$)		White cell count cells per mm^3 ($\times 10^3$)	
Experiment No.	Group	Supplement per kilo of diet	14 days	28 days	14 days	28 days
3	10	None	1.0	1.6	18.6	12.6
3	11	1 mg PGA	2.0	2.4	24.9	19.9
3	12	1 ” ” + 10 g antagonist	1.7	1.8	11.7	6.0

noted on the basal diet. These included slow growth, poor feathering and low hemoglobin content of the blood. When pteroylglutamic acid was added to the diet at an adequate level the growth was rapid and the birds were normal in appearance. Addition of the antagonist together with a suboptimal level of pteroylglutamic acid resulted in growth slower than that obtained with the basal diet, but with a higher level of pteroylglutamic acid the depressing effect of the antagonist on growth was completely reversed. When the antagonist was added alone, all the birds were dead by 3 weeks. Hemoglobin determinations in one experiment indicated the production of anemia by the antagonist and its reversal by pteroylglutamic acid. These results are summarized in Table III.

Summary. Mice on a purified diet with added succinylsulfathiazole developed no signs of pteroylglutamic acid deficiency within 6 weeks. When a crude synthetic preparation of a pteroylglutamic acid antagonist was added to the diet a syndrome appeared which was characterized by slow growth, anemia and leucopenia. Pteroylglutamic acid prevented the appearance of the syndrome. The development of pteroylglutamic acid deficiency in chicks on a purified diet was aggravated by adding the antagonist to the diet, and the effects of the antagonist were reversed by pteroylglutamic acid.

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Venezuela—Pi-Suñer A.

AUTHORS' INDEX

VOLUME 65

(The numerals indicate the page)

Aaron, T. H. and Abramson, H. A. Histamine whealing inhibition, pyribenzamine. 272.
Abramson, H. A. 272.
Agnew, S., Kaplan, M. and Spink, W. W. Staphylocoagulase, penicillin, streptomycin inhibition. 38.
Alexander, B., Lorenzen, E., Hoffmann, R., and Garfinkel, A. Plasma carotene and vitamin A, mineral oil. 275.
Alvord Jr., E. C. 83.
Anderson, R. G. 218.
Applegarth, A. P. 256.
Attyah, A. M. 266.

Ball, G. H. 278.
Bellows, J. C., Burkholder, M. M., Farmer, C. J. Streptomycin, ocular infections. 17.
Bennett, L. L., Applegarth, A. P. and Li, C. H. Diabetes enhancement adrenocorticotropic hormone carbohydrate free diet. 256, 317.
Berman, R. L. 180.
Beyer, K. H. 215.
Bier, O. G. 66.
Blaskovic, D., and Salk, J. E. Vaccine influenza, standardization. 352.
Blecha, E. 227.
Bondy, P. K., and Sheldon, W. H. Liver glycogen demonstration, diabetic acidosis. 68.
Brazda, F. G. 1.
Briller, S. 63.
Brown, H. R., Jr., and Pearson, R. Heart, cardiac output, O_2 consumption relation. 307.
Brush, B. E. 60.
Bryant, J. M., and Blecha, E. Hypertensive vascular heart disease, low Na-forced fluid. 227.
Bucher, K. Phosphorus metabolism, nerves. 24.
Burkholder, M. M. 17.

Cabell, C. A. and Ellis, N. R. Vit. A deficient diets bioassay. 117.
Calder, R. M. Nicotinic acid, myocardial systole, coronary flow, arrhythmias. 76.
Campbell, G. S. 297.
Carr, J. C. Saccharic acid, K salt feeding. 189.
Chaplin, H., Jr. Digitoxin toxicity, DCA. 194.
Chase, H. F. 13.
Chen, K. K. 218.
Chittum, J. R. 95.
Clapper, W. E. Lactobacilli metabolism, NaF. 333.
Clark, W. G. 202.
Clift, M. E. 9.
Code, C. F. 33.
Combs, G. F. 273.
Cook, R. J. 336.
Cooper, G. R. 187.
Correll, J. W. 83.
Coulson, R. A., and Brazda, F. G. Pyridine derivatives, high protein diet. 1.
Cowley, L. L., and Brush, B. E. Wound healing tantalum oxide. 60.
Cruz, W. O., and Ubatuba, F. Blood picture castrate hamster. 321.

Daniel, L. J., Combs, G. F., Norris, L. C., and Heuser, G. F. Bone deformity, chick. 273.
Davis, M. E., and Fugo, N. W. Hormones, sex, early pregnancy, pregnandiol excretion. 283.
De Lamater, J. N. and Hallman, F. A. *Endamoeba histolytica* culture. 26.
Dennis, C., and Eddy, F. D. Colitis ulcerative, vagotomy. 306.
Dick, G. F., and Schwartz, W. B. Hypertension, rice and fruit juice diet. 22.
Dillon, M. L., Cooper, G. R., and Menkin, V. Leukocytosis-promoting factor electrophoresis. 187.
Donovick, R. 107.
Drill, V. A. 192.
Dubin, I. N. *Plasmodium vivax,* exoerythrocytic forms. 154.
Duffy, C. E. 123.
Dulaney, A. D., and Packer, H. *Granuloma inguinale,* complement fixation, pus antigen. 254.
Dutcher, J. D. 326.

Eddy, F. D. 306.
Eisen, H. N., Mayer, M. M., Moore, D. H., Tarr, R., and Stoerk, H. C. Blood serum antibodies, γ-globulin, adrenal cortex. 301.
Ellis, N. R. 117.
Elvehjem, C. A. 5, 97, 115.
Emmart, E. W. Tuberculostasis, 3-n-amylcyclopentane-carboxylic acid derivatives. 156.
Epstein, M. 364.
Erickson, T. C. 115.
Evans, H. M. 317.

Farmer, C. J. 17.
Favour, C. B. Lymphocyte lysis, tubercle b. products. 269.
Fellows, E. J. Circulatory action, phenylpropylamine derivatives. 261.
Finnegan, J. K., Larson, P. S., and Haag, H. B. Cigarette smoke irritation, nicotine. 200.
Foreman, R. C. Shock, hemorrhagic, residual blood redistribution, lethal bleeding vol. 29.
Fowell, D. M. 266.
Fox, D. L. 92.
Francis, T., Jr. Virus influenza, serologic variations. 143.
Franklin, A. L., Stokstad, E. L. R., and Jukes, T. H. Pteroylglutamic acid deficiency acceleration, antagonist. 368.
Fraser, L. E. 178.

Friedheim, E. A. H., and Berman, R. L. BAL, arsenical trichomonacides. 180.
Fruton, J. S. 196.
Fugo, N. W. 283.

Garfinkel, A. 275.
Geissman, T. A. 202.
Gellhorn, A. 83, 221.
George, W. L. 364.
Gilson, W. E. 115.
Ginder, D. R., Matumoto, M., Schlesinger, R. W., and Sabin, A. B. Virus, Jap. B. encephalitis, neutralizing complement fixing antibodies. 130, 135, 183.
Giunti, V. 23.
Goldie, H. *M. tuberculosis* culture Dubos medium. 210.
Goldner, M. G., and Gomori, G. Alloxan diabetes, mechanism, pancreatectomy. 18.
Gomori, G. 18.
Gordan, G. S., Bennett, L. L., Li, C. H., and Evans, H. M. Hormone, growth, low protein diet. 317.
Griffith, G. C. 102.
Gross, L. Cancer, mouse mamma filtrate hemolytic. 292.
Groupe, V. 326.
Guterman, H. S. Oligospermia arginine. 176.

Haag, H. B. 200.
Hadley, M. F. 324.
Haley, T. J., Clark, W. G., and Geissman, T. A. "Vitamin P," capillary bed. 202.
Hall, E. M. 102.
Hallman, F. A. 26.
Hamilton, J. B. 51.
Hamilton, W. F., Attyah, A. M., Fowell, D. M., Remington, J. W., Wheeler, N. C., and Witham, A. C. Heart, simultaneous ventricle ejection? 266.
Hanawalt, V. M., Link, R. P., and Sampson, J. Carbohydrate tolerance test, intrav. 41.
Handley, C. A., and La Forge, M. Mercurial diuresis, thiols. 74.
Hawn, C. vZ. 309.
Heidelberger, M. 66.
Heinle, R. W. 364.
Heller, C. G., Jungck, E. C. Ovarian growth regulation, estrogen inhibition, stimulation by gonadotrophin. 152, 148.
Herman, E. F. 97.
Hesser, F. H. 95.
Hesseltine, H. C. 15.
Heuser, G. F. 273.
Hiestand, W. A., Hadley, M. F., Mercer, S. E., and Sandock, B. K. Blood sugar, hemoglobin, starvation, central depressants. 324.
Hill, Jr., H. C. 95.
Hobby, G. L., and Lenert, T. F. Streptomycin action *in vitro*. 242, 235.
Hobby, G. L., and Lenert, T. F. Streptomycin, *Eberthella typhosa*. 249.
Hoffman, R. 275.
Hogeboom, G. H., Schneider, W. C., and Pallade, G. E. Mitochondria isolation. 320.
Holman, H. R., White, A., and Fruton, J. S. Serum peptidase, adrenal cortex. 196.
Honorato, R. Prothrombin, thrombin time det. 41.
Hooker, C. W., Drill, V. A., and Pfeiffer, C. A. Liver estrogen inactivation failure. 192.
Hoyt, R. E. 70.
Hunter, F. R., and Larsh, H. W. Hemolysis, "control" rbc. 49.
Huntington, R. W. 102.

Johnstone, D. B., Waksman, S. A. Streptomycin II, new species. 294.
Jukes, T. H. 368.
Jungck, E. C., Heller, C. G. and Nesson, W. O. Hormone, gonadotrophic regulation, inhibition, inactivation? 148, 152.

Kaplan, M. 38.
Kirschbaum, A., and Lu, C. S. Leukemia, myelogenous, leucocyte maturation, urethane. 62.
Knowles, R. G. 102.
Kossman, C. E., and Briller, S. Oxigram, cardio-respiratory reserve. 63.
Krueger, R. A. 9.
Kurland, A. A. 348.

La Forge, M. 74.
Larsh, H. W. 49.
Larson, P. S. 200.
Leavitt, Jr., M. D., and Code, C. F. Benadryl, skin anesthesia. 33.
Lehman, W. L. 220, 221.
Lenert, T. F., and Hobby, G. L. Streptomycin action *in vitro*. 235, 242, 249.
Levine M., and Silver, G. B. Colchicine mitosis, cancer. 54.
Levine, M. G., and Hoyt, R. E. Scleroma: complement fixation test. 70.
Leymaster, G. R., and Ward, T. G. Virus, mumps, isolation. 346.
Li, C. H. 256, 317.
Lindquist, J. N. 44.
Link, R. P. 41.
Lorenzen, E. 275.
Lu, C. S. 62.

McAleer, D. S. 172.
McDermott, W. 163.
McKee, C. M., Dutcher, J. D., Groupe, V., and Moore, M. Antibacterial lipids, *Tetrahymena geleii*. 326, 107.
McLaren, B. A., Herman, E. F., and Elvehjem, C. A. Trout nutrition. 97.
McLaren, C. S. 172.
McQuarrie, I., Ziegler, M., and Moore, I. H. Calcium enriched meat, milk Ca, P, Protein sources. 120.
Magill, T. P. 233.
Marinelli, L., and Giunti, V. Hypoglycemia, intraspinal glucose injection. 23.
Matumoto, M. 130, 135.
Mayer, M. M., Osler, A. G., Bier, O. G., and Heidelberger, M. Complement fixation, quantitative. 66, 301.
Melampy, R. M. Erythrocyte amino acids. 213.
Meiklejohn, G., Simpson, T. W., and Stacy, I. B. Virus, encephalitis, Jap. B., domestic animal infection. 359.
Melnick, J. L., and Penner, L. R. Virus poliomyelitis, exp'l. fly infection. 342.
Menkin, V. 187.

Mercer, S. E. 324.
Meyer, K., Gellhorn, A., Prudden, J. F., Lehman, W. L., and Steinberg, A. Colitis, ulcerative, lysozyme. 221.
Meyer, K., Prudden, J. F., Lehman, W. L., and Steinberg, A. Ulcer peptic, gastric lysozyme. 220.
Meyer, O. O. 298.
Miller, B. S. 258.
Miller, K. 222.
Moore, D. H. 301.
Moore, F. J., Ridge, G. K., Huntington, R. W., Hall, E. M., Griffith, G. C., and Knowles, R. G. Heart, rheumatic-like lesion, expl. 102.
Moore, G. E., and Campbell, G. S. Vascular diseases, peripheral, intra-arterial fluorescein. 297.
Moore, I. H. 120.
Moore, M. 326.

Naumann, H. N. Urobilinogen, cerebrospinal fluid. 72.
Nelson, W. O. 148.
Neter, E. Pyribenzamine benadryl chick embryo, serum induced vascular phenomenon. 90.
Newell, G. W., Erickson, T. C., Gilson, W. E., and Elvehjem, C. A. Electroencephalogram, wheat gluten diet. 115.
Newman, H. W., and Yee, J. Alcohol metabolism, electrically induced convulsions. 122.
Norris, L. C. 273.

Osler, A. G. 66.

Packer, H. 254.
Paff, G. H., Rubin, L., and Hamilton, J. B. Perfusion pump. 51.
Pallade, G. E. 320.
Pansy, F. E. 107.
Pearson, R. 307.
Pennell, R. B., Smith, W. E., and Werkheiser, W. C. Hemoglobin sol. containing reducing enzymes. 295.
Penner, L. R. 342.
Persike, E. C., and Ureen, H. J. Rutin, induced proteinuria. 339.
Pfeiffer, C. A. 192.
Porter, K. R., and Hawn, C. vZ. Cell culture, thrombin-fibrinogen clots. 309.
Postlethwait, R. W., Hesser, F. H., Chittum, J. R., and Hill Jr., H. C. Gastric motility, vagotomy. 95.
Prudden, J. F. 220, 221.

Rake, G., McKee, C. M., Pansy, F. E., and Donovick, R. Streptomycin B characteristics. 107.
Remington, J. W. 266.
Richardson, A. P., Walker, H. A., and Miller, B. S. Pentaquine, cardiovascular system. 258.
Richtmeier, A. J., Spooner, M., and Meyer, O. O. Purpura, diethylstilbestrol, α-tocopherol. 298.
Ridge, G. K. 102.
Rose, H. M., and Gellhorn, A. Virus, influenza, inactivation, S, N mustards. 86.
Rosenblum, H., and Fraser, L. E. Rheumatic fever, acute, p-aminobenzoic acid. 178.
Rubin, L. 51.
Rubinstein, H. S., and Kurland, A. A., Electroencephalogram, minimal convulsive electric dosage. 348.
Ruegamer, W. R. 5.
Russo, H. F., Wright, L. D., Skeggs, H. R., Tillson, E. K., and Beyer, K. H. Renal clearance, threonine, phenylalanine. 215.

Sabin, A. B. Encephalitis, Jap. B. mouse brain vaccine, serological response Japanese. 127, 130.
Sabin, A. B., and Duffy, C. E. Encephalitis, Jap. B. uncentrifuged vaccine, antibody response, age. 123.
Sabin, A. B., Ginder, D. R., Matumoto, M., and Schlesinger, R. W. Encephalitis, Jap. B mouse brain vaccine, serological response Japanese. 135.
Sabin, A. B., Schlesinger, R. W., and Ginder, D. R. Virus encephalitis, Jap. B infections. 183.
Salk, J. E. 352.
Sampson, J. 41.
Sandock, B. K. 324.
Sano, M. E. Lymphoma heterologous transmission. 229.
Scheer, B. T., and Fox, D. L. Marine organisms attachment, petrolatum surfaces. 92.
Schlesinger, R. W. 130, 135, 183.
Schmidt, J. L., and Chase, H. F. Muscular activity, curarization. 13.
Schneider, W. C. 320.
Schudmak, M., and Hesseltine, H. C. Penicillin absorption, vagina. 15.
Schultz, E. W. *Pseudomonas aeruginosa* gelatinous variant. 289.
Schwartz, W. B. 22.
Seevers, M. H. 113.
Sharpe, G. 364.
Sheldon, W. H. 68.
Sherman, W. C. Vit. A, carotene, G.I. stability, xanthophyll protective. 207.
Sherrod, T. R. Heart muscle, electrolytes, digitalis. 89.
Shultz, S. 163.
Silver, G. B. 54.
Simpson, T. W. 359.
Skeggs, H. R. 215.
Smith, W. E. 295.
Smolen, J., McAleer, D. S., and McLaren, C. S. Penicillium filtrate, antidotal properties. 172.
Somogyi, M., and Cook, R. J. Blood sugar, fat intake. 336.
Scudi, J. V., Clift, M. E., and Krueger, R. A. Bacitracin, absorption, excretion. 9.
Spink, W. W. 38.
Spooner, M. 298.
Sporn, E. M., Ruegamer, W. R., and Elvehjem, C. A. Growth, reproduction, synthetic rations. 5.
Stacy, I. B. 359.
Steinberg, A. 220, 221.
Stevenson, L. D., Alvord, Jr., E. C., and Correll, J. W. Streptomycin, 8th cranial nuclei necrosis. 83.
Stoerk, H. C. 301.
Stokstad, E. L. R. 368.

Sugg, J. Y., and Magill, T. P. Virus, influenza, reinfection convalescent ferrets. 233.

Tarr, R. 301.
Tillson, E. K. 215.
Tocantins, L. M., and Londquist, J. N. Thromboplastic activity, urine. 44.
Tompsett, R., Shultz, S., and McDermott, W. Penicillin activity, protein-binding. 163.

Ubatuba, F. 321.
Ureen, H. J. 339.

Verwey, W. F., and Miller, K. Pneumococcus, typhoid infections, penicillin, caronamide. 222.

Waksman, S. A. 294.
Walker, H. A. 258.
Ward, T. G. 346.
Weiss, E. D., and Ball, G. H. *Tritrichomonas foetus* nutrition, protein digests. 278.
Welch, A. D., Heinle, R. W., Sharpe, G., George, W. L., and Epstein, M. Pteroylglutamic acid, hematopoietic effect, extrinsic, intrinsic factors. 364.
Welles, J. S., Anderson, R. C., and Chen, K. K. Strophanthidin esters. 218.
Wells, J. H. Blood pressure, $NaNO_2$, organic "nitrates." 161.
Werkheiser, W. C. 295.
Wheeler, N. C. 266.
White, A. 196.
Witham, A. C. 266.
Woods, L. A., Wyngaarden, J. B., and Seevers, M. H. Amidone addiction potentialities. 113.
Wooley, J. G. Niacin deficiency, tryptophane. 315.
Wright, L. D. 215.
Wyngaarden, J. B. 113.

Yee, J. 122.

Ziegler, M. 120.

SUBJECT INDEX

VOLUME 65

(The numerals indicate the page)

- **Adrenal** cortex, blood γ-globulin, antibodies. 301.
  - peptidase. 196.
- **Alcohol** metabolism, electrically induced convulsions. 122.
- **Alloxan** diabetes mechanism. 18.
- **Amidone** addiction potentialities. 113.
- **Antibacterial** lipids, *Tetrahymena geleii.* 326.
- **Arginine,** oligospermia. 176.

- **Bacitracin** absorption, excretion. 9.
- **BAL,** arsenical trichomonacides. 180.
- **Benadryl,** skin anesthesia. 33.
- **Blood** erythrocyte amino acids. 213.
  - picture, castrate hamster. 321.
  - plasma carotene, vit. A, mineral oil. 275.
  - pressure, NaNO$_2$, organic "nitrates." 161.
  - serum antibodies γ-globulin, adrenal cortex. 301.
  - sugar, fat intake. 336.
    - hemoglobin starvation, central depressants. 324.
- **Bone** deformity, chick. 273.

- **Calcium** enriched meat, milk, Ca, P, protein sources. 120.
- **Cancer,** mitotic response colchicine. 54.
  - mouse mamma filtrate, hemolyzes rbc. 292.
- **Carbohydrate** tolerance test, intrav. 41.
- **Castration,** blood picture. 321.
- **Cell** culture, thrombin-fibrinogen clots. 309.
- **Cigarette** smoke irritation, nicotine. 200.
- **Circulatory** action phenylpropylamine derivatives. 261.
- **Colchicine** mitosis, cancer. 54.
- **Colitis,** ulcerative, lysozyme. 221.
  - vagotomy. 306.
- **Complement** fixation, quantitative. 66.
- **Curarization,** muscular activity. 13.

- **Desoxycorticosterone,** digitoxin toxicity. 194.
- **Diabetes** enhancement, adrenocorticotropic hormone, cbhy-free diet. 256.
- **Digitoxin** toxicity, DCA. 194.

- **Electroencephalogram,** minimal convulsive electric dosage. 348.
  - wheat gluten diet. 115.
- **Encephalitis,** Jap. B, centrifuged vaccine, antibody response, age. 123.
  - mouse brain vaccine, serological response Japanese. 135.
  - uncentrifuged vaccine, antibody response, age. 127.
- **Endamoeba histolytica** culture. 26.
- **Eye** infections, streptomycin. 17.

- **Gastric** motility, vagotomy, drugs. 95.
- **Granulosa inguinale** complement fixation, pus antigen. 254.
- **Growth,** reproduction, synthetic diet. 5.

- **Hemoglobin** sol'n containing reducing enzymes. 295.
- **Hemolysis,** "control" rbc. 49.
- **Heart,** cardiac output, O$_2$ consumption relation. 307.
  - muscle electrolytes, digitalis. 89.
  - myocardial systole, coronary flow, nicotinic acid. 76.
  - rheumatic-like lesion exp'l. 102.
  - simultaneous ventricle ejection? 266.
- **Histamine** whealing inhibition, pyribenzamine. 272.
- **Hormone** adrenocorticotropic, diabetes. 256.
  - estrogen inactivation, liver. 192.
  - gonadotrophic regulation; inhibition, inactivation? 148.
  - growth, low protein diet. 317.
  - sex, early pregnancy, pregnandiol excretion. 283.
- **Hypertension,** rice and fruit juice diet. 22.
  - vascular heart disease, low Na-forced fluid. 227.
- **Hypoglycemia,** intraspinal glucose injn. 23.

- **Lactobacilli** metabolism, Na F. 333.
- **Leucocytosis-promoting** factor electrophoresis. 187.
- **Leukemia,** myelogenous, leucocyte maturation, urethane. 62.
- **Liver,** estrogen inactivation failure. 192.
  - glycogen demonstration, diabetic acidosis. 68.
- **Lymphocyte** lysis, tubercle b. products. 269.
- **Lymphoma,** heterologous transmission. 229.
- **Lysozyme,** stomach, peptic ulcer. 220.
  - ulcerative colitis. 221.

- **Marine** organism attachment, petrolatum surface. 92.
- **Mercurial** diuresis, thiols. 74.
- **Mitochondria** isolation. 320.
- **Muscular** activity, curarization. 13.

- **Nerve** activity, P metabolism. 24.
- **Nicotine,** cigarette smoke irritation. 200.

- **Oligospermia,** arginine. 176.
- **Ovarian** growth regulation, estrogen inhibition, stimulation by gonadotrophin. 152.
- **Oxigram,** cardiorespiratory reserve measure. 63.

Penicillin absorption, vagina. 15.
  activity, protein-binding. 163.
  caronamide, pneumococcus, typhoid. 222.
  filtrate, antidotal property. 172.
Pentaquine, cardiovascular system. 258.
Peptidase, adrenal cortex. 196.
Perfusion pump. 51.
Plasmodium vivax, exoerythrocytic forms? 154.
Pneumococcus, typhoid infections, penicillin, caronamide. 222.
Pregnancy, pregnandiol excretion, sex hormones. 283.
Proteinuria, rutin. 309.
Prothrombin, thrombin clotting time det'n. 41.
Pseudomonas aeruginosa, gelatinous variant. 289.
Purpura, diethylstilbestrol, α-tocopherol. 298.
Pyribenzamine, benadryl chick embryo, serum-induced vascular phenomenon. 90.
Pyridine derivatives, high protein diet. 1.

Renal clearance, threonine, phenylalanine. 215.
Rheumatic fever, acute, p-aminobenzoic acid. 178.
Rutin, induced proteinuria. 309.

Saccharic acid, K salt feeding. 189.
Scleroderma, complement fixation test. 70.
Shock, hemorrhagic, residual blood redistribution, lethal bleeding vol. 29.
Staphylocoagulase, penicillin, streptomycin inhibition. 38.
Streptomycin action *in vitro*. 235, 242.
  B characteristics. 107.
  Eberthella typhosa. 249.
  8th cranial nuclei necrosis, degeneration. 83.
  ocular infections. 17.
  II, antibiotic, new species. 294.
Strophanthidin esters. 218.

Thromboplastic activity, urine. 44.
Trichomonacide, arsenical, BAL. 180.
Tritrichomonas fetus nutrition, protein digests. 278.
Trout nutrition. 97.
Tuberculosis, M. culture, Dubos medium. 210.
  bacterial products lymphocyte lysis. 269.
Tuberculosis, 3-n-amyl-cyclopentane-carboxylic acid derivatives. 156.
Typhosa, E., streptomycin. 249.

Ulcer peptic, gastric lysozyme. 220.
Urobilinogen, cerebrospinal fluid. 72.

Vaccine, influenza, standardization. 352.
Vascular disease, peripheral, intra-arterial fluorescein. 297.
Virus, encephalitis, Jap. B, domestic animal infection. 359.
    infections. 183.
    neutralizing, complement-fixing antibodies. 130.
  influenza, inactivation, S, N mustards. 86.
    reinfection convalescent ferrets. 233.
    serologic variations. 143.
    vaccine standardization. 352.
  mumps, isolation. 346.
  poliomyelitis, exp'l. fly infection. 342.
Vit. A, carotene G.I. stability, xanthophyll protective. 207.
    deficient diets, bioassay. 117.
    mineral oil ingestion. 275.
  α-tocopherol, diethylstilbestrol purpura. 298.
  niacin deficiency, tryptophane. 315.
    myocardial systole, coronary flow, arrhythmias. 76.
  P capillary bed. 202.
  PABA, acute rheumatic fever. 178.
  pteroylglutamic acid deficiency acceleration, antagonist. 368.
      hematopoietic effect extrinsic, intrinsic factors. 364.

Wound healing; tantalum oxide. 60.